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d) encodes an antigenic polypeptide having at least 12 amino acid residues; or

e) is synthetically produced.

REMARKS

Status of the Application; and the Present Amendment

Claims 1-17 are pending and stand rejected in the application. With entry of this amendment, claims 8 and 11 have been canceled without prejudice, and claims 1, 3, 4, 10, 16, and 17 have been amended. Claims 3, 16, and 17 have been amended for improved clarity or to correct typographical errors. Claims 1, 4, and 10 have been amended to delete certain claim elements or to recite larger numbers of contiguous nucleotides (i.e., narrowing claim scope). Support for the amendment is provided in the specification, e.g., at Col. 13, lines 7-19. No new matter has been introduced by the amendments.

The following remarks addresses issues raised in the instant Office Action.

Claim Objections

Claim 17 was objected to because of repetition of the verb "is." Applicant has herewith amended the claim by deleting the redundant wording.

Claims 1, 3, 4, 8-11, 13, and 17 were objected to because some claim amendments previously presented were in bold font. The Examiner requested that clarification be made with respect to the significance of the bold font. In response, Applicant points out that the bond type was used to highlight words added and was merely for ease of reference. All currently pending claims are reproduced below without using bold font for inserted words.

Rejections under 35 U.S.C. 101 and 35 U.S.C. 112, 1st Paragraph

Claims 1-17 are rejected under 35 U.S.C. 101 as allegedly lacking apparent or disclosed patentable utility. The claims are also rejected under 35 U.S.C. 112, first paragraph, on the same grounds. Specifically, the Examiner acknowledges that the application has disclosed a novel isolated DNA encoding a protein and the protein encoded by the DNA. However, the Examiner says that the specification does not disclose the biological role of the

protein or its significance. The Examiner states that the claimed IL-B30 sequences have been isolated because of its similarity to known proteins, but that sequence-to-function methods of assigning protein function are prone to errors. The Examiner further states that until some actual and specific significance can be attributed to the IL-B30 protein, the invention is incomplete. The Examiner concludes that there is no immediately obvious patentable uses for the IL-B30 molecules. This rejection is respectfully traversed for the reasons stated below.

Applicant notes that the Commentary on the Utility Examination Guidelines, Federal Register, 66 (4) 1092-1099, at 1096 (Jan. 5, 2001), states that "[w]hen a class of proteins is defined such that the members share a specific, substantial, and credible utility, the reasonable assignment of a new protein to the class of sufficiently conserved proteins would impute the same specific, substantial, and credible utility to the assigned protein." Rejecting suggestions to adopt a per se rule rejecting homology-based assertions of utility, the Commentary further states that:

> A patent examiner must accept a utility asserted by an applicant unless the Office has evidence or sound scientific reasoning to rebut the assertion. The examiner's decision must be supported by a preponderance of all the evidence of record. In re Oetiker, 977 F.2d 1443, 1445, 24 USPQ2d 1443, 1444 (Fed. Cir. 1992). More specifically, when a patent application claiming a nucleic acid asserts a specific, substantial, and credible utility, and bases the assertion upon homology to existing nucleic acids or proteins having an accepted utility, the asserted utility must be accepted by the examiner unless the Office has sufficient evidence or sound scientific reasoning to rebut such an assertion. [Utility Examination Guidelines, Federal Register 66 (4) at page 1096; emphasis added]

As detailed below, Applicant has disclosed patentable utility in the present application that is substantial, specific, and credible. In addition, the Office only cites to a few references which suggest that in general homology-based assignment of function may not always be accurate. However, no concrete evidence specific to IL-B30 was provided in the Office Action to rebut or contradict Applicant's teachings. To the contrary, following the present disclosure, peer reviewed reports later confirmed and experimentally verified the

utilities disclosed in the subject specification. Further, there is additional real-world uses of the claimed sequences that would have been readily apparent to the skilled persons.

1. The specification discloses patentable utility

In the instant case, Applicant has provided in the specification the amino acid sequences and DNA sequences encoding human and other mammalian IL-B30 cytokines. The specification also disclosed the structural similarities between IL-B30 and other members of the long chain cytokines, e.g., members of the IL-6 family of cytokines. The IL-6 cytokine family includes cytokines such as IL-6, IL-11, IL-12, and G-CSF (see, e.g., page 21, lines 18-21). It was well know in the art that the these cytokines share certain similar functions in mediating cellular activities. Thus, due to their structural similarities with IL-B30, one would reasonably conclude that IL-B30 would be involved in similar biological activities.

The present invention has disclosed patentable utilities that are certainly substantial, specific, and credible. For example, the specification disclosed that IL-B30 could act as functional or receptor antagonists, e.g., binding to cytokine receptors and regulating immune responses mediated by the cytokine receptors (see, e.g., pages 15, lines 1-8), and that the different cytokine receptor complex could share certain components with an IL-B30 receptor complex (page 50, lines 19-25). From these disclosures, it would be readily apparent to a skilled artisan that IL-B30 polypeptides and nucleic acids can be useful for screening for modulators of the other cytokines (e.g., members of the IL-6 cytokine family) and their receptors. In addition, as taught in the specification, IL-B30 can also be useful to identify and isolate novel cytokine receptors (pages 49-50). Besides the biological functions of IL-B30 as disclosed in the specification, the present inventors further taught that IL-B30 can be useful in the treatment of abnormal medical conditions, including immune disorders, e.g., T cell immune deficiencies, chronic inflammation, or tissue rejection, or in cardiovascular or neurophysiological conditions (see, e.g., pages 15, lines 8-15; and pages 40-41).

The Office cites to Doerks et al., Brenner et al., and Bork et al. in maintaining the instant rejections. However, these references at most suggest that, in general, function assignment based on sequence homology may not always be accurate. Unlike what is required

under the Utility Guidelines to sustain a lack of utility rejection, these references do not provide scientific evidence that is specific to IL-B30 in order to dispute or contradict the utilities disclosed in Applicant's invention. Applicants further notes that the Office apparently takes the position that only actual experimental data on the disclosed activities of IL-B30 would satisfy the utility requirement. However, such is not the legal test for patentable utility that is illustrated by the Commentary on the Utilities Guidelines quoted above.

2. The Specification details how to make use of the disclosed utilities

In addition to the patentable utilities discussed above, the present disclosure also provided guidance for one to make use of and experimentally confirm the disclosed utilities. Thus, the present disclosure is not limited to the hypothetical functions of IL-B30 based on its homology to other cytokines, as alleged in the Office Action. Rather, the present invention also disclosed specifically how to make use of the utilities. For example, Applicant has taught specifically how to assay expression level of IL-B30 in various human and mouse cell types, e.g., macrophages, Th1 cells, and dendritic cells (see, e.g., pages 53-59). The specification also taught that elevated expression levels of IL-B30 detected with these assays would indicate that IL-B30 plays a role in inflammation, a regulation or effector role in T helper subsets, particularly Th1 immune responses, and a role in antigen presentation or germinal center T or B cell interactions with DC (see, page 56, lines 23-29). The specification further taught how to experimentally examine other biological functions of IL-B30 as disclosed in the present invention (pages 61-64). For example, the specification provides detailed procedures for one to assay effects of IL-B30 on cytokine production (e.g., page 62) or peripheral blood mononuclear cell proliferation (e.g., page 63). Significantly, as discussed below, employing substantially the same the assays and procedures as disclosed in the specification, later published studies demonstrated experimentally the various biological functions of IL-B30.

3. Published reports confirmed the disclosed utilities by following the present disclosure

A number of post-filing publications confirmed and experimentally verified the utilities of the subject invention. For example, these studies found that IL-30B, now termed

IL-23 p19 subunit, associates with subunits of other known cytokines, binds to cytokine receptors, and regulates immune responses. Specifically, Oppmann et al. (Immunity, 13: 715-25, 2000, copy attached) reported that IL-B30 is structurally and evolutionarily related to the other long chain cytokines (see, e.g., Figure 1) and also showed their functional similarities. Specifically, this reference reported that IL-23, composed of IL-B30 and IL-12p40, has certain biological activities similar to IL-12 (a member of the IL-6 cytokine family). It demonstrated that activated dendritic cells secrete detectable levels of IL-23, that IL-23 binds to IL-12 receptor and also activates Stat4 in PHA blast T cells, and that IL-23 stimulates IFN-gamma production and proliferation in PHA blast T cells, as well as in CD45RO (memory) T cells. Similarly, Wiekowski et al., J. Immunol., 166: 7563-70, 2001 (copy attached) reported that IL-B30 plays a role in multiorgan systemic inflammation, runting, infertility, and premature death in transgenic mice. The data reported in this reference confirm that IL-B30 shares biological properties with IL-6, IL-12, and G-CSF (see, e.g., Table III on page 7569), as taught in the present invention.

It is important to note that, in confirming utilities of the present invention, these published studies all employed the same or similar methods and assays as described in the subject specification. As such, Applicant submits that these post-filing publications provide additional support that the utilities of IL-B30 as disclosed in the subject specification are indeed specific and credible.

4. Additional real-world utilities that would have been apparent

In addition to the above-discussed utilities specifically set forth in the specification, the skilled artisans would readily appreciate the other credible, substantial, and real-world utilities of the present invention. For example, the novel polynucleotide sequences identified by the present inventors can be readily applied in polynucleotide array technology. Polynucleotide arrays are commercially available and have been widely used by the skilled persons in the art. Such arrays typically contain oligonucleotide or cDNA probes to allow detection of large numbers of mRNAs within a mixture. They are often used to study

differential gene expression and to analyze candidate drugs for roles in modulation of a disease state.

Thus, the IL-B30 sequences are useful for inclusion on a GeneChip™ array or the like together with probes containing a variety of other genes. With increased diversity of probe sequences, the modified arrays provide improved tools for the various applications of polynucleotide arrays. For example, such arrays can be used for expression monitoring, especially during cell differentiation or other related cellular processes. The modified arrays can be used to compare expression profiles between different tissues or between different conditions of the same tissue (healthy vs. diseased or drug-treated vs. control) to identify differentially expressed transcripts. The differentially expressed transcripts are then useful, e.g., for diagnosis of disease states, or to characterize responses of drugs. No one would doubt that such applications of the present invention constitute credible, substantial, and real-world utilities.

For all of the reasons stated above, it is submitted that the present invention has disclosed patentable utilities that are substantial, specific, and credible. Accordingly, Applicant respectfully requests that the instant rejection be withdrawn.

Rejections under 35 U.S.C. 112, second paragraph

Claim 3 is rejected as allegedly indefinite in the recitation of "or" and "and." The claim has been amended herewith. The amended claim now makes it clear that the claimed sequences encode "c" and hybridize to "a" or "b".

Claim 16 is rejected as allegedly vague and indefinite because it is not clear whether all the limitations are together or in the alternative. In response, Applicant has amended claim 16 by adding "or" at the end of claim element (c). Such amendment makes it clear that claim elements (a)-(d) are in the alternative.

Applicant thanks the Examiner for his careful reviewing of the claims. In light of the above-discussed claim amendments and clarifications, Applicant submits that there is no indefiniteness in the presently pending claims. Withdrawal of the rejections under 35 U.S.C. 112, second paragraph, is respectfully requested.

CONCLUSION

In view of the foregoing, Applicant believes all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 650-326-2400.

Respectfully submitted,

Hugh Wang
Reg. No. 47,163

Attachments: Marked-up version of all pending claims
Wiekowski et al., J Immunol, 166: 7563-70, 2001
Oppmann et al. (Immunity, 13: 715-25, 2000

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Appendix. Marked-up Version of All Pending Claims
(claims unamended herewith appear in small font)

1. (Twice Amended) An isolated or recombinant polynucleotide encoding an antigenic polypeptide comprising:

a) at least [40] 132 contiguous amino acids from a mature coding portion of SEQ ID NO: 2;

b) at least 17 contiguous amino acids from a mature coding portion of SEQ ID NO: 4; or

c) at least [17] 30 contiguous amino acids from a mature coding portion of SEQ ID NO: 5.

2. The polynucleotide of claim 1, encoding a mature polypeptide of:

a) SEQ ID NO: 2;

b) SEQ ID NO: 4; or

c) SEQ ID NO: 5.

3. (Amended) The polynucleotide of claim 1, which encodes amino residues 155-164 of SEQ ID NO: 2 and hybridizes under stringent wash conditions of at least 65°C., less than about 150 mM salt to the complement of:

a) the open reading frame of SEQ ID NO; 1; or

b) the open reading frame of SEQ ID NO: 3 [; and

c) encodes amino residues 155-164 of SEQ ID NO: 2].

4. (Twice Amended) An isolated or recombinant polynucleotide encoding an antigenic polypeptide comprising [:

a) at least 67 contiguous nucleotides of a coding portion of SEQ ID NO: 1, wherein said contiguous nucleotides are from nucleotides 466-555 of SEQ ID NO: 1; or

b)] at least 67 contiguous nucleotides of a coding portion of SEQ ID NO: 3, wherein said contiguous nucleotides are from nucleotides 580-670 of SEQ ID NO: 3.

5. A recombinant or expression vector comprising said polynucleotide of claim 1.

6. An isolated host cell comprising said expression vector of claim 5.

7. A method of making an antigenic polypeptide comprising expressing said recombinant polynucleotide of claim 1 and isolating said polypeptide, thereby making said antigenic polypeptide.

9. Said polynucleotide of claim 1b), wherein said contiguous amino acids number 20, 30, 35, or 40.

10. (Twice Amended) Said polynucleotide of claim 1c), wherein said contiguous amino acids number [20, 30,] 35, or 40.

12. Said polynucleotide of claim 2, that is a variant due to the degeneracy of the genetic code.

13. The polynucleotide of claim 3, wherein said wash conditions are
a) at least 70°C.;
b) less than about 100 mM salt; or
c) both a) and b).

14. The polynucleotide of claim 3, wherein said wash conditions
a) are at least 50% formamide;
b) are less than about 100 mM salt; or
c) are both a) and b).

15. The polynucleotide of claim 1, that:
a) encodes the mature polypeptide of SEQ ID NO: 2, 4, or 5; or
b) comprises the mature coding portion of SEQ ID NO: 1 or 3.

16. (Amended) The polynucleotide of claim 2, wherein said polynucleotide:

a) encodes a polypeptide with a natural sequence of the mature coding portion of SEQ ID NO: 2 or 4;

b) is isolated from nature;

c) encodes a polypeptide comprising five or fewer conservative substitutions from a natural sequence of SEQ ID NO: 2 or 4; or

d) encodes a polypeptide comprising five or fewer conservative substitutions from a natural sequence of SEQ ID NO: 4.

17. (Amended) The polynucleotide of claim 3, which [is]:

a) is attached to a solid substrate;

b) is detectably labeled;

c) is in a sterile composition;

d) encodes an antigenic polypeptide having at least 12 amino acid residues; or

e) is synthetically produced.

Immunity, Vol. 13, 715–725, November, 2000, Copyright ©2000 by Cell Press

Novel p19 Protein Engages IL-12p40 to Form a Cytokine, IL-23, with Biological Activities Similar as Well as Distinct from IL-12

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Summary

A novel sequence discovered in a computational screen appears distantly related to the p35 subunit of IL-12. This factor, which we term p19, shows no biological activity by itself; instead, it combines with the p40 subunit of IL-12 to form a novel, biologically active, composite cytokine, which we term IL-23. Activated dendritic cells secrete detectable levels of this complex. IL-23 binds to IL-12Rβ1 but fails to engage IL-12Rβ2; nonetheless, IL-23 activates Stat4 in PHA blast T cells. IL-23 induces strong proliferation of mouse memory ($CD4^+CD45Rb^{low}$) T cells, a unique activity of IL-23 as IL-12 has no effect on this cell population. Similar to IL-12, human IL-23 stimulates IFN-γ production and proliferation in PHA blast T cells, as well as in CD45RO (memory) T cells.

Introduction

The proliferation, differentiation, and effector functions of immune cells are regulated in part by a network of soluble protein factors. Discrete families of these molecules have been identified by sequence and structural analysis. Within the diverse set of sampled protein folds, the four-α helix bundle topology uniquely distinguishes members of the hematopoietic cytokine family (Rozwarski et al., 1994). Among the members of this large family, a further clustering is evident. Within these subfamilies, members often display faint but significant stretches of amino acid similarity that are indicative of a closer evolutionary and functional relationship.

A particularly important subfamily of helical cytokines encompasses a set of factors that are related to interleukin-6 (IL-6). These now include IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), cardiotrophin-1 (CT-1), ciliary neurotrophic factor (CNTF), and novel-neurotrophin-1 (NNT-1). This group of cytokines exhibits a wide range of often overlapping biological functions that are transmitted via multichain cell surface receptors, which are typically formed by high-affinity, cytokine-specifi receptor chains and lower-affinity, signal-transducing chains. The presence of shared signal-transducing receptors offers an explanation for the overlapping functions of IL-6-like cytokines.

Two other IL-6-like cytokines, G-CSF and the p35 subunit of IL-12, signal through private superfamily receptors. Within this outlier group, G-CSF is a biologically active protein that binds directly to a specific, signal-transducing G-CSFR. By contrast, IL-12p35 is not active on its own and instead forms part of a composite factor with a chain known as p40 (Kobayashi et al., 1989). Whereas p35 shows homology to the IL-6-type cytokines and G-CSF, p40 is a soluble member of the cytokine receptor superfamily. Only the composite p35p40 IL-12 molecule was found to display biological activity, although a p40 homodimer may function as an IL-12 antagonist by competing for the IL-12 receptor (Gately et al., 1996).

IL-12 plays a critical role in cell-mediated immunity (Gately et al., 1998; Trinchieri, 1995, 1998). Its activities are triggered through a high-affinity receptor complex that gathers two closely related subunits, IL-12Rβ1 and β2 (Chua et al., 1995; Presky et al., 1996b). Although no evidence exists for IL-12 receptor promiscuity, the p35 subunit has been suggested to bind to a second soluble cytokine receptor called EBI3 (Devergne et al., 1997). However, no biological activity has been reported for the p35-EBI3 pair. By contrast, phenotypic differences between IL-12 p40-deficient mice and IL-12 p35-deficient mice with respect to the clearance of bacterial infections (Decken et al., 1998; Brombacher et al., 1999) invite the possibility that p40 is involved in functional complexes with molecules other than p35.

In this report, we describe a novel helical cytokine that was identified by searching sequence databases with a computationally derived profile of IL-6 subfamily structures. This protein was designated p19, representing its approximate molecular weight. We further show that p19 is part of a novel composite factor that consists of a disulfide-bridged complex between p19 and the p40 subunit of IL-12. This novel p19p40 complex is naturally expressed by activated mouse and human dendriti cells and has biological activities that are similar to but distinct from IL-12. These activities result from interaction of the p19p40 complex with IL-12Rβ1 and an additional, novel receptor subunit.

Results

p19 Was Identified Computationally

We searched sequence databases with a computationally derived profile (Gribskov et al., 1987) of members of the interleukin-6 (IL-6) helical cytokine family. This search led to identification of a novel cytokine, which we named p19. The p19 cDNA sequences encode 189/196 amin acid polypeptides (human and mouse, respectiv ly) corresponding to mature proteins with calculated molecular weights of 18.7 and 19.8 kDa. Both proteins contain fiv cystein residues and n N-glycosylati n sites. Human and mouse p19 are 70% id ntical and most

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†These authors contributed equally to this work.

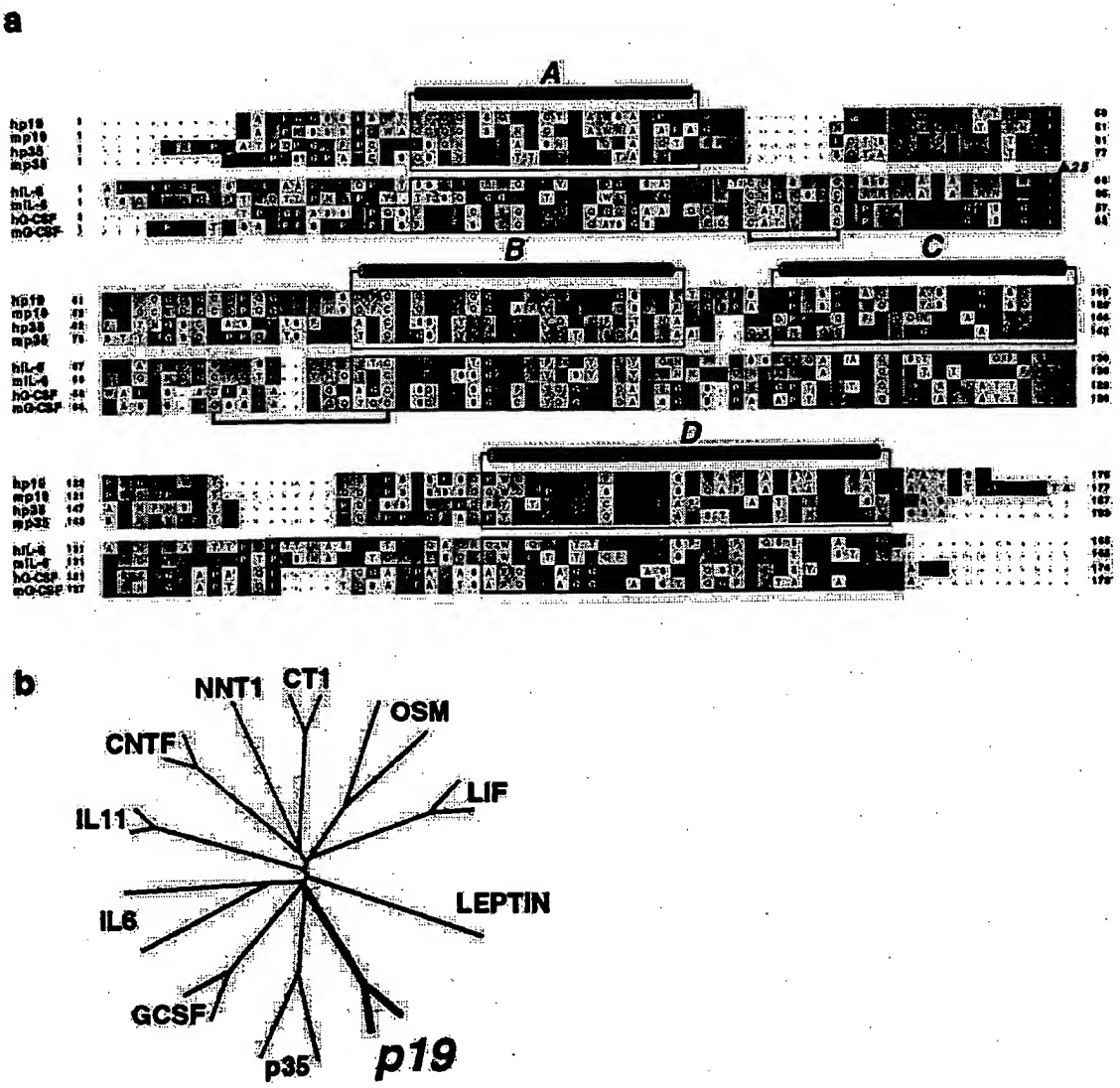

Figure 1. Alignment of Amino Acid Sequences for Selected Members of the Long Chain α-Helical Cytokine Family

(A) Sequences for all cytokines are shown as mature peptides (human p35, GenBank accession number B38957; mouse p35, NP_032377; human IL-6, P05231; mouse IL-6, P08505; human G-CSF, NP_000750; and mouse G-CSF, NP_034101). The alignment is based on the overlapping structures of IL-6 (PDB entry code 1il6) and G-CSF (PDB entry code 1rhg). The α helices of hIL-6 and hG-CSF are boxed according to their secondary structures. The four helices predicted for p19 and p35 are labeled A, B, C, and D. The two disulfide bonds in IL-6 and G-CSF are numbered 1 and 2. Closed triangle indicates the position of 25 additional amino acids in human and mouse p35, not shown in the alignment, but that likely pack against the helix bundle. The amino acid coloring scheme depicts chemically similar residues: green (hydrophobic), red (acidic), blue (basic), yellow (C), orange (aromatic), black (structure breaking), and gray (small).

(B) Evolutionary dendrogram of selected cytokines, showing the branching pattern that captures the various IL-6-like subgroups. The tree is rooted by leptin.

closely related to IL-12p35, IL-6, and G-CSF (Figure 1A). An evolutionary dendrogram shows p19 and IL-12p35 as closest neighbors (Figure 1B).

p19 and IL-12p40 Form a Novel C mp site Factor

The presence of a signal peptid suggested that p19 would be secreted wh n xpressed in mammalian cells. However, upon tran fecti n of 293T cell with tagged f rms f human r mouse p19, only a small amount of mouse (Figure 2B, lan 2) but n t the human protein (Figure 2A, lane 2) could be immunoprecipitated from the supernatant. Both proteins could be detected in the cellular lysates of transfected cells, indicating inefficient secretion (data not shown). mp19 transfection supernatant or semipurified mp19 protein was inactive when analyzed in various bioassays (data not shown). W next investigated the possibility that p19 is part of a composit factor like IL-12 (Gubler et al., 1991; Wolf et al., 1991) and requires coexpression with a partner protein to be ffici ntly secreted. p19 was coexpressed with various nonsignaling receptors of the IL-6 family, including

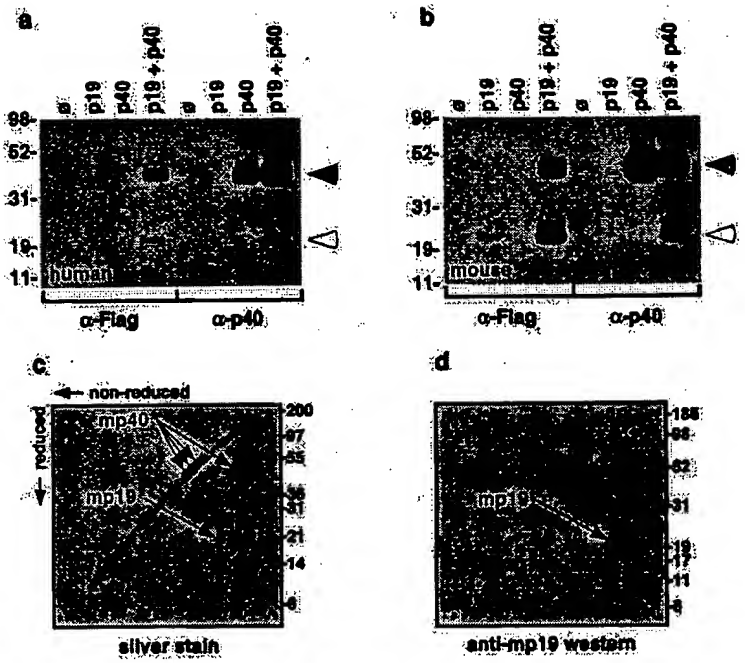

Figure 2. IL-12p40 and p19 Form a Soluble Disulfide-Linked Heterodimer

(A and B) 293T cells were transiently transfected with empty vector (lanes 1 and 5) or expression vectors for p19-Flag (lanes 2 and 6), IL-12 p40 (lanes 3 and 7), or both (lanes 4 and 8). Twenty-four hours after transfection, cells were metabolically labeled for 16 hr, and proteins were immunoprecipitated from supernatant with anti-Flag agarose or p40-specific antibodies coupled to protein G sepharose. Immunoprecipitation of the human (A) and the mouse (B) p19p40 complex.

(C and D) Nonreduced followed by reduced SDS-PAGE on mouse p19p40 supernatant purified from adenovirus coinfected 293T cells by heparin chromatography. (C) Silver stained gel. (D) Anti-mp19 Western blot. Molecular weight markers are indicated in kDa.

tagged forms of EBI3, soluble IL-6R, NR6 (CLF-1), and IL-12p40. Among these molecules, only the coexpression of p19 and IL-12p40 led to enhanced secretion of p19 (Figure 2, lanes 4 and 8). Moreover, both proteins were coimmunoprecipitated with antibodies against either tagged p19 or p40 (anti-Flag or anti-p40, respectively), indicating that p19 and IL-12p40 form a soluble cytokine/receptor complex similar to that of IL-12 p35p40. A small amount of mp19 migrates at a slightly higher molecular weight (Figure 2B). The higher band could result from O-linked glycosylation. Several potential O-linked glycosylation sites are predicted (Hansen et al., 1998). To investigate whether p19p40 is a disulfide-linked heterodimer like IL-12, the complex was purified from adenovirus p19 and p40 coinfected 293 cells and analyzed on two-dimensional SDS gels. Silver staining of the gel showed the presence of p19p40 disulfide-linked heterodimer in addition to free p40 monomers and dimers (Figure 2C). The identity of the mouse p19 spot on the silver stained gel was confirmed by anti-mp19 Western blot (Figure 2D).

Natural p19p40 Is Expressed by Activated Dendritic Cells

Expression analysis of mouse cDNA libraries showed the presence of p19 mRNA in various tissues and cell types (Figure 3A). Highest mRNA levels were found in polarized Th1 cells and activated macrophages. We could not detect p19 mRNA in bone marrow–derived dendritic cells. In contrast, both mouse and human dendritic cells derived from peripheral blood monocytes express high levels of p19 mRNA (Figure 3B). M nocyte-derived dendritic cells cultured in GM-CSF and IL-4 and activated via CD40 f r 2 days also express high levels of p40 mRNA and to a lesser ext nt p35 mRNA (Figure 3B). W analyzed natural p19p40 in th supernatants f activated mouse and human dendritic cells. Mouse and human dendritic cells derived from peripheral blood adherent cells were stimulated with GM-CSF and IL-4 for 5 days. After culture with TNFα, LPS, and anti-CD40 antibody for 2 days, the culture supernatant was harvested. A native mouse p19p40 complex could be detected in a sandwich ELISA (Figure 4A) as well as by SDS-PAGE/anti-mp19 Western blot (Figure 4B). Similarly, a native human p19p40 complex was immunoprecipitated from activated human DC with anti-hp40 mAb. The precipitate was resolved on IEF/SDS-PAGE 2D gels (Figure 4D) and compared to the patterns obtained with similar precipitates from specific 293T cell cotransfection experiments (Figure 4C).

p19p40 Specifically Acts on Mouse Memory T Cells

To investigate the biological effects of the p19p40 complex, we initially engineered a soluble tagged fusion protein by flexibly linking the mouse p40 chain to mouse p19 (Hy-p40-p19). This chimeric molecule follows the design of the "hyper-IL-6" cytokine (IL-6Rα-IL-6, (Fischer et al., 1997) that shows greater solution stability, presumably because the cytokine and its cognate receptor domains are closely tethered and form a composite binding surface for the cellular receptors. A similar p40-p35 fusion protein shows specific activity identical to native IL-12 (Anderson et al., 1997). For our biological studies, we have used both mouse and human Hy-p40-p19 proteins. Experiments were also performed with conditioned medium of p19p40 cotransfected cells or adeno coinfected cells with identical results. First, we investigated the role of the Hy-p40-p19 on $CD4^+$ T cell subsets in comparison to IL-12. Naiv T cells ($CD4^+CD45RB^{high}$) and memory/activated T cells ($CD4^+CD45RB^{low}$) were sorted from IL-10-deficient mice, which have elevated levels of memory T cells compared to wild-type mice (Davidson et al., 2000). The T cell subsets were stimulated with anti-CD3 mAb for 5 days

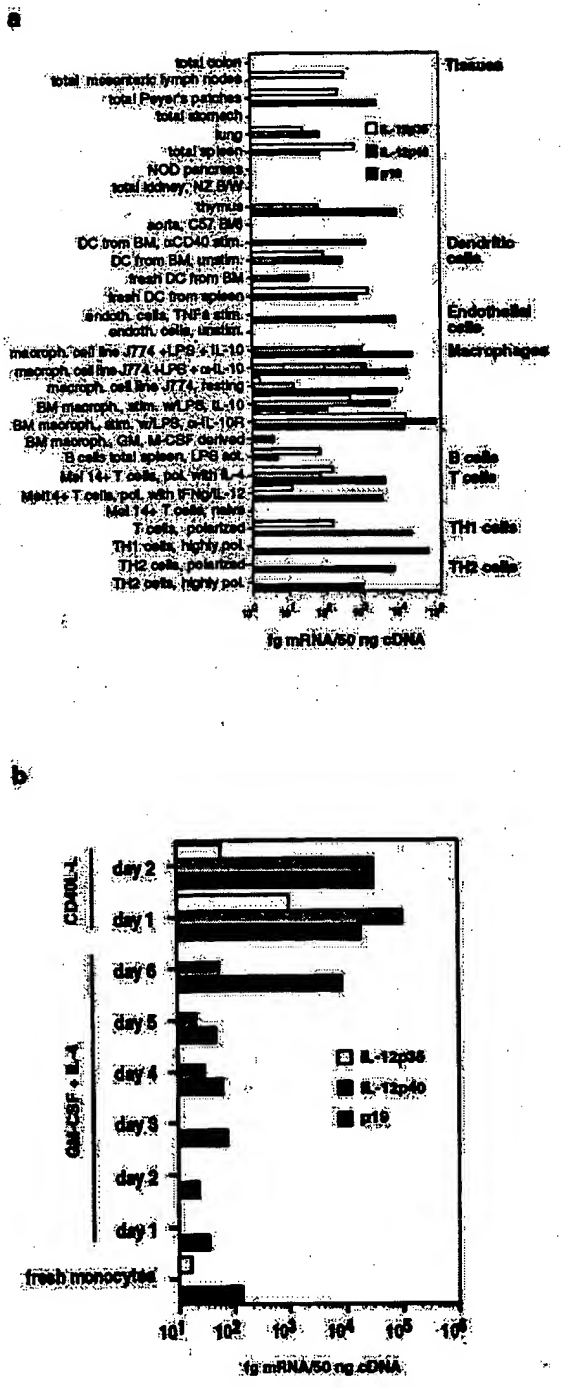

Figure 3. Distribution of Mouse p19, IL-12p35, and IL-12p40 mRNA in Various Tissues and Cell Types

(A) Quantitative PCR on various mouse libraries. (B) Quantitative PCR on human cultured dendritic cells. Dendritic cells were derived from human monocytes cultured in GM-CSF and IL-4 for 6 days and activated for 2 days in coculture with CD40L transfected L cells. Total RNA was isolated and analyzed for expression of p19, IL-12p40, and IL-12p35 mRNA by using the Taqman technique. mRNA levels are expressed in fg per 50 ng of cDNA.

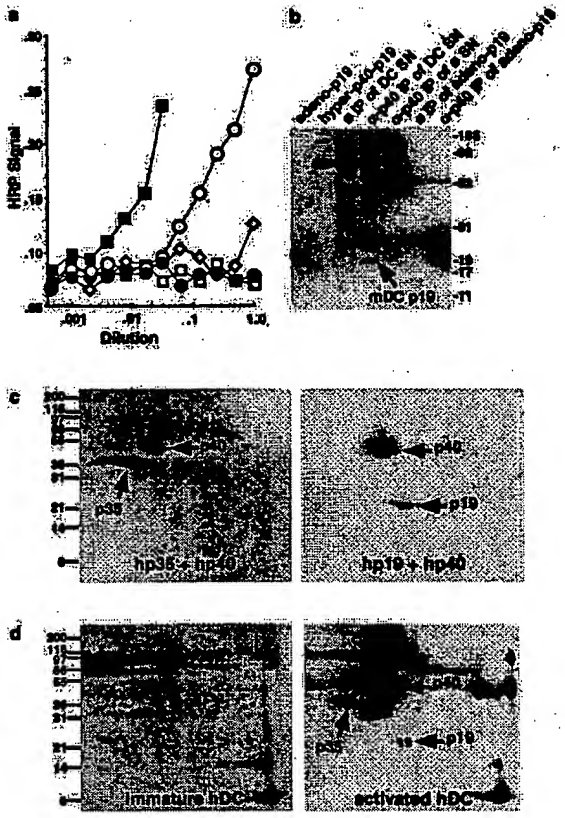

Figure 4. Natural p19p40 Heterodimer in Activated Mouse and Human Dendritic Cell Supernatants

DC supernatants and control medium were concentrated prior to detection by ELISA or immunoprecipitation (IP).

(A) Sandwich ELISA on activated (open circles) and immature (closed circles) mouse DC supernatant. Medium (open squares), 1000 ng/ml IL-12 (open diamonds), and 100 ng/ml Hy-p40-p19 (closed squares) were used as controls. Plates were coated with mp19-specific mAb 20C10. Bound cytokine was detected with biotinylated anti-mp40 mAb and HRP-streptavidin.

(B) Detection of mouse DC-produced p19 by Western blot. Control medium and DC supernatant were treated with rat anti-mp40 and isotype control beads, respectively. Blotted protein was detected with rat anti-mp19 mAb 10A11 and HRP-conjugated anti-rat antibody.

(C) IEF/SDS-PAGE 2D gels of human IL-12 p35p40 (left) and p19p40 (right) from 293T cotransfected cells.

(D) Human immature (left) and activated (right) dendritic cell supernatants.

in the presence of anti-IL-2 mAb and various amounts of IL-12 or Hy-p40-p19. Upon stimulation in the presence of IL-12, naive ($CD4^+CD45RB^{high}$) but not memory T cells ($CD4^+CD45RB^{low}$) proliferated (Figure 5A). In contrast, Hy-p40-p19 protein had no effect on naive T cells but strongly induced proliferation of memory T cells. The stimulatory effect on the memory T cells was 100-fold lower in the presence of a neutralizing anti-IL-12p40 antibody. Th sam proliferative signal could be induced by stimulation with conditioned medium of p19p40 cotransfected cells (Figure 5B). Similar results were obtained with memory T cells from wild-type mice; however, their response to Hy-p40-p19 was always less than

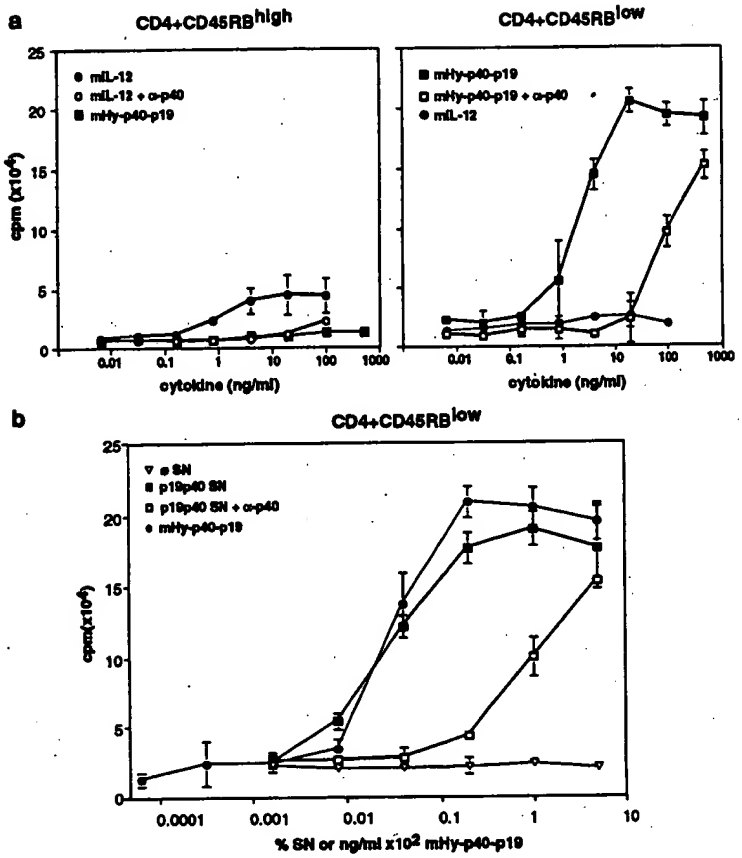

Figure 5. p19p40 Stimulates the Proliferation of Mouse Activated/Memory T Cells but Not Naive T Cells

(A) Sorted $CD4^+CD45RB^{high}$ or $CD4^+CD45RB^{low}$ T cells were cultured with plate-bound anti-CD3 mAb. Anti-IL-2 mAb (100 μg/ml) was added to all cell cultures. Mouse IL-12 or purified mouse Hy-p40-p19 was titrated into the cultures, ± anti-IL-12(p40) mAb (250 μg/ml). [3H]TdR incorporation (counts per minute) was assessed after 5 days of culture. Data are mean ± SD of triplicate wells and are representative of three experiments.

(B) Sorted $CD4^+CD45RB^{low}$ similarly treated. Purified mouse Hy-p40-p19 (started at 500 ng/ml) or supernatant from 293T cells cotransfected with p19 and p40 (started at 5% by volume) was titrated into the culture, ± anti-IL-12(p40) mAb (250 μg/ml). [3H]TdR incorporation (counts per minute) was assessed after 5 days of culture. Data are mean ± SD of triplicate wells.

the response of T cells from IL-10-deficient mice (data not shown).

p19p40 Acts on Human Memory and Naive T Cells

We next analyzed the ability of human Hy-p40-p19 to induce proliferation and IFN-γ production in human PHA blast T cells. Hy-p40-p19 significantly enhanced the production of IFN-γ by 7-day-old PHA blasts activated by plate-bound anti-CD3 and soluble anti-CD28 mAbs (Figure 6A). The maximal levels of IFN-γ production induced by saturating amounts of Hy-p40-p19 were lower compared to those induced by saturating amounts of IL-12. The IFN-γ enhancing effects of both Hy-p40-p19 and IL-12 could be blocked by anti-IL-12Rβ1 or anti-p40/p70 antibodies. However, the anti-p35 antibody only blocked the IFN-γ production induced by IL-12 and not that induced by Hy-p40-p19. The low level IL-4 production by PHA blasts was not affected by Hy-p40-p19 or IL-12 (data not shown). Hy-p40-p19 and IL-12 enhanced the proliferation of PHA blasts (Figure 6B). Again, the effects of Hy-p40-p19 on proliferation could be blocked by anti-IL-12Rβ1 or anti-p40/p70 but not by anti-p35 antibodies.

We also compared the activity of Hy-p40-p19 and IL-12 on CD45RA (naive) and CD45RO (m mory) T cell populations isolated from PBMC. FACS-sorted CD45RA or CD45RO T cells were activated by plate-b und anti-CD3 antibodies and solubl anti-CD28 mAbs in th absence r presence of Hy-p40-p19, IL-12, or IL-2. Activated CD45RA T cells did not produce IFN-γ in the absence of Hy-p40-p19 or IL-12, whereas CD45RO T cells produced moderate amounts at day 3 and day 6 (Figure 6C). Addition of Hy-p40-p19 to these cultures did not enhance IFN-γ production by CD45RA T cells at 3 days after activation but caused a modest increase in IFN-γ production at day 6. In contrast, Hy-p40-p19 enhanced IFN-γ production by CD45RO T cells at both day 3 and day 6 after activation. Addition of IL-12 slightly enhanced production of IFN-γ by CD45RA T cells at day 3 but induced a significant increase at day 6, which is in agreement with the previously described requirement for upregulation of IL-12Rβ2 expression on naive T cells (Rogge et al., 1997). In addition, IL-12 significantly enhanced the production of IFN-γ by CD45RO T cells at both day 3 and day 6. Expression of IFN-γ was in all cases strongly dependent on the endogenous production of IL-2 since neutralization with anti-IL-2 and anti-IL-2Rα mAbs abolished it completely (data not shown). Neither Hy-p40-p19 nor IL-12 induced IFN-γ production by CD45RA or CD45RO T cells in the absence of activation by anti-CD3 and anti-CD28 mAbs (data not shown).

Similar to the experiments in the mouse, Hy-p40-p19 induced a more pronounced effect on proliferation of CD45RO T cells as compared to CD45RA T cells at day 6 after activation in the absence of ndogenous IL-2 (Figure 6D). However, both CD45RA and CD45RO T cells could respond to IL-12 under these conditions. These results indicate that Hy-p40-p19 acts on human CD45RO

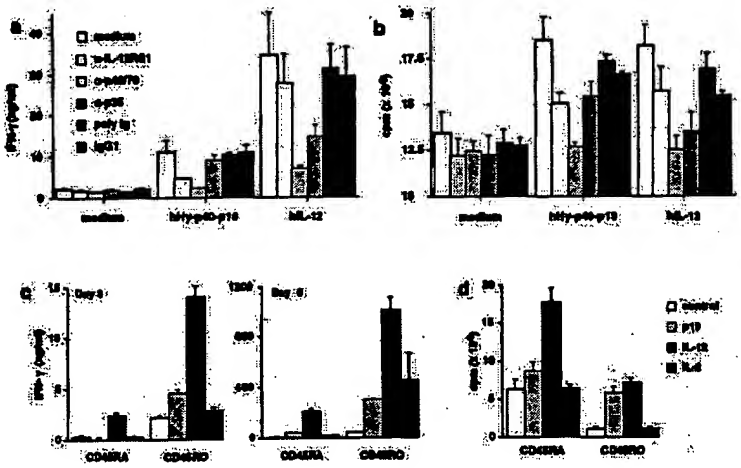

Figure 6. p19p40 Stimulates the IFN-γ Production and the Proliferation of Human PHA Blasts and Activated/Memory T Cells

(A) PHA blasts were derived from cultured PBMC in PHA (0.1 μg/ml)- and IL-2 (10 U/mL)-containing medium. After 7 days, cells were stimulated for 60 hr with 40 ng/ml hHy-p40-p19 or 1 ng/ml IL-12 and 1 μg/ml soluble anti-CD28 in 96-well plates coated with 10 μg/ml anti-CD3 mAb. IFN-γ was measured by ELISA.

(B) Remaining cells were then pulsed with 1 μCi/well ^{3}H-thymidine (NEN) for 6 hr, and incorporation of ^{3}H-thymidine was determined by scintillation counting. All blocking antibodies and isotype controls were used at 10 μg/ml.

(C and D) p19p40 has a more pronounced effect on human CD45RO (memory) than on CD45RA (naive) T cells. FACS-purified CD45RA and CD45RO T cells were cultured on anti-CD3 (10 μg/ml) and anti-CD28 (1 μg/ml) coated 96-well plates and stimulated with 40 ng/ml hHy-p19-p40, 1 ng/ml IL-12 (R&D Systems), or 100 U/ml IL-2 (R&D Systems) for 60 or 136 hr. IFN-γ production (C) and proliferation (D) were determined as in (A) and (B).

m mory T cells and on CD45RA naive T cells but on the latter population only after a prolonged activation period.

Hy-p40-p19 Binds to IL-12Rβ1 but Not to IL-12Rβ2

To investigate the molecular basis of the effects described above, we analyzed the signaling receptors used by p19p40. Ba/F3 cells were infected with retroviral constructs encoding human or mouse forms of IL-12Rβ1 or -β2 (Gubler and Presky, 1996; Wu et al., 1996) (Figure 7). Ba/F3 cells expressing hIL-12Rβ1 bound human Hy-p40-p19 and hIL-12 (Figure 7A). Binding of mouse Hy-p40-p19 could be detected only on cells expressing the IL-12Rβ1 but not on cells expressing the IL-12Rβ2 (Figures 7B and 7C). Ba/F3 cells tranfected with both receptor subunits proliferated in response to IL-12 but not upon addition of Hy-p40-p19 stimulation (Figure 7D). Taken together, our data show that p19p40 and IL-12 share IL-12Rβ1 but not IL-12Rβ2 in their respective signaling complexes.

Hy-p40-19 Activates Stat4

IL-12 stimulation results in the activation of Stat4 (Bacon t al., 1995; Jacobson et al., 1995). IL-12Rβ2 has been identified as the subunit that links IL-12 signaling to Stat4 activation (Naeger et al., 1999). Since p19p40 does not share this receptor with IL-12 but presumably uses its own unique receptor subunit in addition to IL-12Rβ1, w asked whether p19p40 can activate Stat4. Hy-p40-p19 significantly induced the activation of Stat4 in human PHA blast T cells (Figure 7E). The level of activation was always less than that observed with IL-12 stimulation, which is in agreement with the lower levels of IFN-γ induced by p19p40 in these cells. This result suggests that p19p40 is linked to the Stat4 signaling pathway. The identification of a specific p19p40 receptor is currently under investigation.

Discussion

We describe a novel four-α h lix cytokin called p19 that is most closely related in structure to IL-12p35. This cytokine is also similar to IL-12p35 in other ways: (1) p19 cosecretes with the p40 subunit of IL-12; (2) formation of biologically active p19p40 heterodimer requires synthesis of both subunits within the same cell; (3) the p19p40 interaction is stabilized by an interchain disulfide bond; and (4) p19p40 binds to IL-12Rβ1, the p40-specific component of IL-12R (Presky et al., 1996a). p19p40 differs from p35p40 in requiring an as yet unidentified transmembrane receptor subunit to complete the p19p40 signaling receptor complex. The identification of a receptor common to both signaling complexes provides a molecular rationale for our finding that p19p40 shows both overlapping and unique functions in comparison with IL-12. The interleukin designation for p19p40 is Interleukin-23.

IL-12 p35p40 is mainly produced by monocytes, macrophages, and other antigen-presenting cells. Similarly, p19p40 is produced by activated human and mouse dendritic cells. In fact, in the supernatant of activated human dendritic cells we find evidence for the simultaneous production of both IL-12 and p19p40 heterodimers (Figures 4C and 4D). Although p19 mRNA is expressed in endothelial cells and polarized T cells, p40 is not found in these cells. The availability of functional IL-12 is also limited by the expression of p40 and not by p35, which is expressed in most cell types at low levels (D'Andrea et al., 1992). This suggests that functional expression of both composite factors is regulated in a similar fashion. p19 has no N-glycosylation sites. In contrast, p35 is extensively posttranslationally modified by N-linked glycosylation. Recent studies show that these modifications of p35 are a required step in the secretion of the p35p40 complex (Carra et al., 2000). Secretion of p40 does not require such modifications. Since secretion of neither p40 nor p19 depends on the addition of N-linked oligosaccharides, it is possible that upon activation of antigen-presenting cells capable of producing both factors p19p40 is produced at earlier times.

Our initial attempts to detect a biological activity for p19 by itself failed. Partially purified mouse p19 had no biological activity on its own when tested in the same

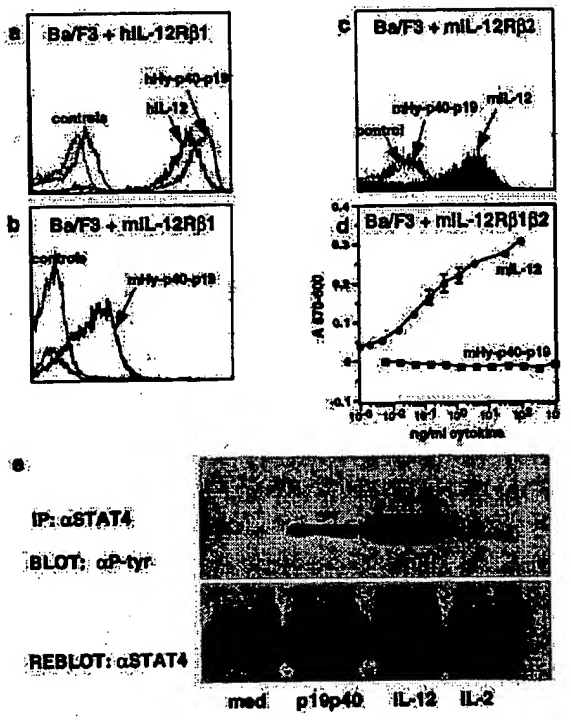

Figure 7. The p19p40 Complex Binds to IL-12Rβ1 but Not to IL-12Rβ2 and Activates Stat4

Cells were infected with retroviral supernatants for 24–48 hr and then incubated with cytokines. Bound Hy-p40-p19 was detected using an anti-Flag mAb, bound IL-12 with anti-p40 mAb.

(A) Ba/F3 cells expressing hIL-12Rβ1 bind human Hy-p40-p19. Controls, no antibody and secondary antibody only; hIL-12, 1 μg/ml; hHy-p40-p19, 1 μg/ml.

(B and C) Ba/F3 cells expressing mIL-12Rβ1 but not mIL-12Rβ2 bind mouse Hy-p40-p19. (B) Controls, Ba/F3-mIL-12Rβ1, no antibody and secondary antibody only; Ba/F3-mIL-12Rβ1, 1 μg/ml mouse Hy-p40-p19. (C) Control, Ba/F3-mIL-12Rβ2, secondary antibody only; mIL-12, 1 μg/ml; mouse Hy-p40-p19, 1 μg/ml.

(D) Ba/F3 cells expressing mIL-12Rβ1 and β2 respond to IL-12 but not Hy-p40-p19. Cells were stimulated with varying cytokine concentrations, and proliferation was measured as metabolic activity visualized by the reduction of a colorimetric REDOX indicator. (Closed circles) and (closed squares), stimulation of Ba/F3-IL-12Rβ1β2 with mIL-12 or mHy-p40-p19, respectively.

(E) Hy-p40-p19 induces phosphorylation of Stat4. Day 3 PHA blasts were incubated with medium, Hy-p40-p19 (1 μg), IL-12 (50 ng), or IL-2 (1000 U) for 20 min and lysed. Stat4 immunoprecipitates were run on denaturing gels, transferred and blotted with anti-phosphotyrosine mAb 4G10, and stripped and reblotted with anti-Stat4 mAb.

in vitro assays that later identified p19p40 as the active complex (data not shown). Additionally, administration of p19 adenovirus to mice did not reveal a biological response. Similarly, transgenic mice expressing mouse p19 under the control of a liver-specific promoter showed no obvious abnormalities (M. Wiekowski, unpublished data). In contrast, transgenic mice expressing mouse p19 mRNA in multiple tissues display a striking phenotype characterized by runting, systemic inflammation, infertility, and premature death (M. Wiekowski et al., unpublished data). Moreover, this phenotype can be transferred to irradiated mice transplanted with p19 transgenic bone marrow cells indicating that hematopoietic cells from the bone marrow produce biologically active p19. Together, these experiments indicate that p19, either administered as protein or expressed via mRNA on its own, is not sufficient to elicit a biological response.

In characterizing the biological activity of p19p40, we initially compared it to IL-12. IL-12 is a cofactor that synergizes with IL-2 to enhance the proliferation, cytotoxicity, and production of cytokines, in particular IFN-γ, by T cells and NK cells (Trinchieri, 1995; Gately et al., 1998). Furthermore, IL-12 induces the differentiation of naive T cells into Th1 cells (Hsieh et al., 1993; Manetti et al., 1993). Like IL-12, p19p40 enhanced proliferation and production of IFN-γ by activated human PHA blast T cells. The maximum levels of IFN-γ production induced by p19p40 were always lower than those induced by IL-12, even at saturating levels of the added cytokines. This characteristic may make p19p40 a more suitable entity to stimulate the cell-mediated immune response in cancer patients, as IL-12 administration leads to severe cytotoxicity associated with extremely high IFN-γ levels in the serum of such patients (Leonard et al., 1997). The immunoregulatory effects of p19p40 were also examined on $CD4^+$ naive and memory T cell subsets in both mice and human. In mice, p19p40 did not enhance proliferation of IL-12-responsive naive CD45RB high cells. Similar observations were made on human CD45RA naive T cells. However, CD45RA T cells could respond to p19p40 by increasing production of IFN-γ after prolonged stimulation. This might be explained if, like IL-12Rβ2, the p19p40- specific receptor subunit is not present on freshly isolated naive T cells but is induced following TCR triggering. In contrast, both mouse and human memory T cells respond strongly to p19p40 by enhanced proliferation and, in the case of human cells, by enhanced IFN-γ production. Interestingly, human memory T cells but not mouse memory T cells were also very responsive to IL-12. This may represent a true species difference or more likely indicates that the subpopulations of T cells as defined by these CD45 isoforms are not equivalent.

IL-12 plays an important role in promoting cell-mediated immunity against microbial pathogens. Endogenous IL-12 is essential for the host defense against *Mycobacterium tuberculosis*, *Leishmania major*, *Listeria monocytogenes*, and *Klebsiella pneumoniae*, as determined by neutralization with blocking anti-IL-12 p40 antibodies (Tripp et al., 1994; Greenberger et al., 1996; Cooper et al., 1997; Mattner et al., 1997). However, these same antibodies, as shown here, also block the activity of p19p40. Thus, studies using anti-p40 antibodies address the role of both IL-12 and p19p40. Analyses of IL-12p35- and IL-12p40-deficient mice have shown that p35-deficient mice are less susceptible to *L. monocytogenes* infection than p40-deficient mice (Brombacher et al., 1999); in addition, IL-12 p40-deficient mice died earlier and developed higher organ burdens following infection by *Cryptococcus neoformans* than p35-deficient mice (Decken et al., 1998). Since p35-deficient mice are unable to produce IL-12 but should still be capable of making p19p40, this observation suggests that p19p40 can contribute to clearance of *Listeria* and *Cryptococcus* and argues for a protective role of p19p40 in bacterial infection. Similarly, the phenotype of IL-

12Rβ1-deficient mice is more severe than that of IL-12Rβ2-deficient mice (Wu et al., 1997, 2000). In particular, IL-12Rβ1 animals are more severely impaired in their ability to produce IFN-γ following and to xin administration in vivo and following ConA or anti-CD3 activation of splenocytes in vitro. Together, these results indicate that elimination of both IL-12 and p19p40 pathways results in a more severe disturbance of the immune system than loss of only IL-12 signaling.

Although IL-12 plays a key role in the generation of protective immunity, it has also been implicated in the immunopathology of organ-specific autoimmune diseases in humans such as diabetes, arthritis, and Crohn's disease. Direct evidence that IL-12 plays a major role in generating Th1-mediated diseases has been provided by rodent studies in which treatment with a neutralizing anti-IL-12 mAb prevents the onset of diseases such as collagen-induced arthritis (Adorini et al., 1997), experimental autoimmune encephalomyelitis (EAE) (Leonard et al., 1995), and chronic IBD (Blumberg et al., 1999). Because antibodies used in these studies block the function of both IL-12 and p19p40, these studies do not address the role of each individual factor in these diseases. Support for a unique role of p19p40 in chronic inflammation comes from our observation that p19p40 has a much more pronounced effect on the proliferation of memory T cells isolated from IL-10-deficient mice with inflammatory bowel disease as compared to memory T cells from wild-type mice or normal human donors. Thus, the greatly increased number of Th1 memory/activated cells present in chronically inflamed intestines and draining lymph nodes of IL-10-deficient mice may reflect their selective expansion in response to p19p40 (Davidson et al., 2000). The predominance of Th1 cells with a memory/activated phenotype has also been observed in chronic lesions of rodents with various forms of autoimmune disease (TNB-induced colitis, EAE, and collagen-induced arthritis). Whether p19p40 plays a critical role in the maintenance and or function of these T cells remains to be determined. Thus, studies are in progress to establish the importance of p19p40 as a mediator of immunological disease and conversely, as a costimulator of protective immune responses.

The discovery of p19p40 as a novel composite factor closely related to IL-12 in structure and with biological activities similar as well as distinct from IL-12 raises important issues. The anti-p40 antibodies used in critical studies to address the role of IL-12 in immunity and immunopathology do not discriminate between IL-12 and p19p40. From the work presented here, as well as from studies based on mice deficient in either shared or unique IL-12 and p19p40 pathway components, evidence is emerging that the biological functions of p19p40 and IL-12 are intimately related. We are currently focusing on the development of reagents to discriminate between the biological functions of p19p40 and IL-12 and their specific contributions to immunity and immunopathology.

Experimental Procedures

Identification of Human p19

A structural alignment of available IL-6 family cytokine folds (CNTF, LIF, IL-6, OSM, and GCSF) from FSSP (Holm and Sander, 1998) was profile aligned to other sequences (including distant species variants of the aforementioned cytokines, plus CT-1, GPA, and viral IL-6's) with Clustal X (Thompson et al., 1997)—with some manual adjustment. A weighted profile (Thompson et al., 1994) of the most conserved region of the fold, the C-terminal D helix segment, an ~40 amino acid block, was created. Fast scans of sequence databases on a Bioccelerator machine (Compugen, Tel Aviv, Israel) with the Profilesearch program (Gribskov et al., 1987) identified two IMAGE ESTs (AA18955 and AA418747) that were used for the computational reconstruction of the human p19 sequence, and the cloning of its full-length cDNA.

Identification of Mouse p19

Based on a strong cross-species hybridization signal, pools of a RAW library (activated monocyte cell line) were generated and screened with a human p19 probe. The full-length 1353 bp cDNA encodes a 189 aa protein. Both human and mouse p19 cDNA sequences have been deposited at GenBank (accession numbers AF301619 and AF301620). Identical sequences were deposited by Y. Hirata and Y. Kosuge (accession numbers NM_016585 and AB030001).

Transient Transfection, Metabolic Labeling, and Immunoprecipitation

Cells (1×10^6) were transiently transfected with 5 μg empty vector or expression vectors encoding human or mouse forms of p19-N-Flag, IL-12p40, or both. Cells were cultured for 24 hr and then metabolically labeled for 16 hr with 50 μCi/ml Pro-mix L-[^{35}S] in vitro cell labeling mix (Amersham Pharmacia) in cysteine/methionine free MEM. Proteins were precipitated from 300 μl supernatant with either the anti-Flag M2 agarose (Sigma), anti-hp40/p70 antibody C8.6 (Pharmingen), or anti-mp40/p70 C17.8 (Genzyme) bound to protein G sepharose (Amersham Pharmacia).

2D-PAGE

For nonreducing/reducing PAGE, purified p19p40 heterodimer was run in two lanes of a nonreducing 10% NuPage gel in MES running buffer (Novex). The lanes were excised, reduced in sample buffer containing DTT, laid horizontally on two-well 10% gels, and run reduced in a second dimension. One gel was silver stained (Daiichi) while the other was blotted to a PVDF membrane and developed using the mouse p19-specific rat mAb 19A11 (1 μg/ml) and the HRP-coupled sheep anti-rat Ig NA932 (Amersham). For isoelectric focusing, immunoprecipitated ^{35}S-labeled heterodimer was run on pH 3–10 immobiline drystrips (Amersham Pharmacia) followed by SDS-PAGE and autoradiography.

Quantitation of mRNA Expression

cDNAs from various libraries or cultured macrophages and dendritic cells were prepared as described (Bolin et al., 1997) and used as templates for quantitative PCR. Fifty nanograms cDNA was analyzed for expression of p19, IL-12p35, or IL-12p40 by the fluorogenic 5′-nuclease PCR assay (Holland et al., 1991) using the ABI Prism 7700 Sequence Detection System (Perkin-Elmer). Primers and probes for human and mouse IL-12p35 and IL-12p40 were obtained as Taqman PDAR's (Perkin-Elmer, Foster City, CA). Analysis of cDNA samples from cultured cells was corrected for expression of 18S rRNA using a VIC-labeled probe (Perkin-Elmer, Foster City, CA) in multiplex reactions.

Expression of mp19 IgG and E Tag Fusion Proteins

A HindIII-XhoI fragment encoding the mp19 protein was inserted into a modified form of pCDM8 encoding a C-terminal hIgG fusion protein. A vector encoding E Tag fusion protein was similarly constructed using pCDM8 (Pharmacia). Proteins were produced via transient transfection of COP5 or 293 cells followed by affinity chromatography purification via Protein A or Anti-E Tag. The Etag fusion protein was blotted onto an PVDF membrane (0.2 μm, Biorad, Hercules, CA) and subjected to Edman degradation. The derived sequence was L-A-V-P-R-S-S-S-P-D, indicating that the mature N-terminal mp19 residue is L20.

Development of Rat Anti-Mouse p19 Monoclonal Antibodies

Rat anti-mouse p19 monoclonal antibodies (mAbs) were produced from splenocytes of a female 8-week-old Lewis rat (Harlan Sprague Dawley, Indianapolis, IN) immunized with mouse p19:Ig fusion protein. The rat was primed with 50 μg of fusion protein in Complete Freund's Adjuvant and boosted three times. Splenocytes were fused with the mouse myeloma P3X63-AG8.653 using PEG 1500 (Boehringer-Mannheim). Hybridoma supernatants were screened in indirect ELISA on PVC plates coated with 50 μl of 0.5 μg/ml p19:Etag fusion protein. Selected positive hybridoma lines were subcloned, grown in serum-free medium supplemented with SITE (Sigma), and purified.

Purification of Natural Mouse and Human p19p40 Heterodimers

Dendritic cell supernatants and control medium were concentrated to 45× using Centriprep 10 concentrators (Millipore). Immunoprecipitations of recombinant and natural human p19p40 heterodimers were performed using goat-anti-mouse Ig beads preloaded with the mouse anti-hp40/p70 antibody C8.6 (Pharmingen), while isotype-control beads contained mouse IgG1 349,040 (Beckton-Dickinson). Mouse p19p40 heterodimers and fusion protein were detected in solution using a sandwich ELISA in which plates were coated with the p19-specific monoclonal antibody 20C10, and detection was performed with the biotinylated p40-specific monoclonal antibody 18482D (Pharmingen) and HRP-streptavidin 016-030-084 (Jackson). Mouse p19 Western blots were performed with the rat-anti-p19 monoclonal antibody 19A11.

Recombinant Adenovirus and Protein Production

m19 cDNA and mIL-12p40 cDNA were inserted separately into the transfer vector pQB1-AdCMV5-GFP (Quantum Biotechnologies, Montreal, Canada) by PCR. Recombinant adenovirus was produced as described in Quantum applications manual 24AL98. Recombinant virus (moi 100 mp19 virus plus moi 1000 mp40 virus) were used to infect 5×10^8 cells in 1 L CMF-1 with culture in a Nunc Cell Factory (Naige Nunc, Naperville, IL) for 3 days. The culture medium was clarified by centrifugation and filtered prior to application to a 1 ml NHS-activated HiTrap (Pharmacia, Uppsala, Sweden) column coupled to 1 mg of mAb 20C10. The column was washed with PBS and eluted with 100 mM glycine (pH 3.0), and the eluate was immediately loaded onto a 4.6 × 100 mm Poros R2/H column (PerSeptive Biosystems, Cambridge, MA) with elution via a 80 ml linear gradient from 20% to 50% acetonitrile/0.1% trifluoroacetic acid.

Expression of Human and Mouse Hy-p40-p19

HindIII-NotI fragments were generated encoding the mature coding sequence of either human or mouse IL-12p40, followed by the synthetic linker GSGSSRGGSGSGGSGGGGSKL and by the mature coding sequence of either human or mouse p19. Fragments were inserted into pFLAG-CMV-1 (Sigma). Proteins were produced via transient transfection of 293 cells followed by affinity chromatography purification via anti-FLAG M2-agarose (Sigma).

Mouse T Cell Proliferation Assay

$CD4^+CD45RB^{high}$ or $CD4^+CD45RB^{low}$ T cell subsets were purified from the spleen and mesenteric lymph nodes of >6- month-old *IL-10*$^{-/-}$ C57/B6 N12 mice as described (Davidson et al., 1998). Cells were fractionated into $CD4^+CD45RB^{high}$ and $CD4^+CD45RB^{low}$ cell populations by two-color sorting on a FACSTAR plus (Beckton Dickinson). All populations were >99% pure upon reanalysis. $CD4^+CD45RB^{high}$ or $CD4^+CD45RB^{low}$ were put into a proliferation assay with plate-bound anti-CD3 (145.2C11) stimulation as described (Davidson et al., 1998). Additions to the growth media included anti-IL-2 mAb (JES6-1A12) 100 μg/ml, anti-IL-12(p40) mAb (C.17.8), 250 μg/ml, IL-12 (R&D), purified recombinant Hy-p40-p19, 293T cell supernatant containing mouse p19p40 heterodimer or mock supernatant. Cells were incubated for 5 days in a humidified chamber (37°C, 5% CO_2) with [^{3}H]TdR (Amersham) added at a final concentration of 1 μCi/well for the last 24 hr of incubation.

Cell Culture

PBMC were isolated from buffy coats of healthy donors (Stanford Blood Bank). Human monocytes were obtained from PBMC by negative selection using Dynabeads M-450 (by Dynal A.S., Oslo. Norway). Purified monocyte populations (85%–90% $CD14^+$) were cultured in GM-CSF (800 U/ml) and IL-4 (300 U/ml) (Schering-Plough, Kenilworth, NJ) at 5×10^5 cells/ml in RPMI + 10% FBS for 6 days, with a change of medium at day 3. At day 6, dendritic cells were induced to mature by coculture with irradiated (7000 rad) CD40 ligand transfected L cells, LPS (1 μg/ml), IFN-γ (100 U/ml), TNFα (100 U/ml) (R&D Systems), or combinations of these.

PHA Blast Generation and Activation

PHA blasts were derived by culture of PBMC in Yssel's medium with 0.1 μg/ml PHA (Wellcome) at 10^6 cells/ml. Cells were plated at a density of 2×10^4 cells per well with or without 40 ng/ml hHy-p19-p40 or 1 ng/ml huIL-12 on a 96-well plate coated with 10 μg/ml anti-CD3 and 1 μg/mL soluble anti-CD28. After 60 hr, IFN-γ production was determined by ELISA. Cells were then pulsed with 1 μCi/well with ^{3}H-thymidine (NEN) for 6 hr, harvested, and incorporation of ^{3}H-thymidine determined. In blocking experiments, the following antibodies were used: polyclonal goat anti-human IL12Rβ1 (R&D cat#AF839), mouse anti-human p40/p70 C8.6 (Pharmingen cat#20510D), mouse anti-human p35 (DIACLONE cat#855.120.010), mouse anti-human p40 (Pharmingen cat#20711D), polyclonal goat Ig (Jackson Immuno Research code 0055-000-003), and monoclonal mouse IgG1 (Pharmingen cat#20800D) as isotype controls at 10 μg/ml. Western blots were performed following immunoprecipitation of PHA blast lysates with anti-stat4 mAb (UBI cat # 06-788) and blotting with anti-phosphotyrosine mAb 4G10 (UBI cat # 05-321) or reblotting with anti-stat4.

CD45RA/CD45RO T Cell Proliferation and IFN-γ Production

FACS-purified CD45RA and CD45RO T cells (purity > 99%) were cultured at a density of 4×10^4 cells/well in a 96-well plate previously coated with anti-CD3 antibody at 10 μg/ml and soluble anti-CD28 at 1 μg/ml with or without 40 ng/mL hHy-p40-p19, 1 ng/mL hIL-12 (R&D Systems), or 100 U/ml hIL-2 (R&D Systems). Anti-hIL-2 mAb 17H12 and anti-hIL-2R Mab B-B10 (Diaclone) were added at 10 μg/ml where indicated. Cells were incubated for 60 or 136 hr, and 100 μl aliquots were collected to determine IFN-γ production by ELISA. Proliferation was measured as described for PHA blasts.

FACS Analysis and Proliferation Assay

Human IL-12Rβ1 cDNA (kindly provided by Dr. Xiaojing Ma) was cloned in the retroviral vector pMX (Kitamura, 1998). Ba/F3 cells were infected with retroviral supernatants for 24–48 hr on petri dishes coated with 30 μg/ml recombinant fibronectin fragments (Retronectin, TaKaRa). Expression of hIL-12Rβ1 was detected using goat anti-hIL-12Rβ1 (R&D Systems) followed by phycoerythrin (PE)-conjugated donkey anti-goat IgG (Jackson Immunoresearch). Mouse IL-12Rβ1 and IL-12Rβ2 cDNAs in pMX-IRES-EGFP were provided by A. O'Garra and L. Showe (Showe et al., 1996; Heath et al., 2000). Cells expressing both receptors were prepared by coinfection with both retroviruses. Hy-p40p19 and IL-12 binding was assessed by FACS. Cells were incubated with 0–10 μg/ml (0–168 nM) Hy-p40p19 or 0–1 μg/ml IL-12 for 30 min on ice, followed by a single wash. Bound Hy-p40p19 was detected using 10 μg/ml anti-FLAG M2 mAb (Sigma) followed by PE-conjugated anti-mouse IgG (Jackson Immunoresearch), or biotinylated M2 (Sigma) followed by streptavidin-PE (Pharmingen). Results were similar using the two procedures. Human IL-12 binding was detected using anti-IL-12p40 mAb C11.5 (Pharmingen) followed by PE-conjugated goat anti-mouse IgG. Mouse IL-12 binding was detected using rat anti-mIL-12 (p40) mAb C17.15 (R&D Systems) followed by goat anti-rat IgG-PE (Jackson Immunoresearch). Proliferation responses were measured as described (Ho et al., 1993).

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GenBank Accession Numbers

The GenBank accession numbers for the human and mouse p19 sequences reported in this paper are AF301619 and AF301620, respectively.

Ubiquitous Transgenic Expression of the IL-23 Subunit p19 Induces Multiorgan Inflammation, Runting, Infertility, and Premature Death

Maria T. Wiekowski,* Michael W. Leach,† Ellen W. Evans,† Lee Sullivan,* Shu-Cheng Chen,* Galya Vassileva,* J. Fernando Bazan,‡ Daniel M. Gorman,‡ Robert A. Kastelein,‡ Satwant Narula,* and Sergio A. Lira[1]*

p19, a molecule structurally related to IL-6, G-CSF, and the p35 subunit of IL-12, is a subunit of the recently discovered cytokine IL-23. Here we show that expression of p19 in multiple tissues of transgenic mice induced a striking phenotype characterized by runting, systemic inflammation, infertility, and death before 3 mo of age. Founder animals had infiltrates of lymphocytes and macrophages in skin, lung, liver, pancreas, and the digestive tract and were anemic. The serum concentrations of the proinflammatory cytokines TNF-α and IL-1 were elevated, and the number of circulating neutrophils was increased. In addition, ubiquitous expression of p19 resulted in constitutive expression of acute phase proteins in the liver. Surprisingly, liver-specific expression of p19 failed to reproduce any of these abnormalities, suggesting specific requirements for production of biologically active p19. Bone marrow transfer experiments showed that expression of p19 by hemopoietic cells alone recapitulated the phenotype induced by its widespread expression, pointing to hemopoietic cells as the source of biologically active p19. These findings indicate that p19 shares biological properties with IL-6, IL-12, and G-CSF and that cell-specific expression is required for its biological activity. *The Journal of Immunology,* **2001, 166: 7563–7570.**

Cytokines comprise a large family of secreted proteins that bind to and signal through defined cell surface receptors on a wide variety of target cells. Many cytokines share structural features and functions during development, immune response, or inflammation. Searching the databases with a computationally derived profile of IL-6, Oppmann et al. (1) have recently identified a novel protein and named it p19. This molecule shares homology with members of the IL-6/IL-12 family of cytokines, which includes IL-6, oncostatin M, LIF, ciliary neurotrophic factor, cardiotrophin-1, novel neurotrophin-1, G-CSF, and p35. IL-6, IL-11, oncostatin M, LIF, ciliary neurotrophic factor, cardiotrophin-1, and novel neurotrophin-1 elicit multiple overlapping biol gical activities by signaling through specific receptors that share gp130 as signal transducer. These biological acitivities include stimulation of acute phase responses, hemopoiesis, thrombopoiesis, osteoclastogenesis, neuronal differentiation and survival, and cardiac hypertrophy (reviewed in Refs. 2–4). In contrast, G-CSF signals independently of gp130 and induces neutrophilic granulocytosis in transgenic mice (5). The last member of this family is p35, a molecule that is itself apparently devoid of biological activity. However, when p35 associates with p40, a soluble member f the cytokine receptor superfamily, it forms a powerful cytokine, IL-12, that induces Th1 differentiation and the release of IFN-γ from Th1 and NK cells (reviewed in Ref. 6).

To investigate the biological properties of p19, we generated transgenic animals expressing it ubiquitously or in a tissue-specific fashion. Phenotypic analysis of these transgenic animals indicates that p19 has biological properties related to IL-6, G-CSF, and IL-12 and that its expression from hemopoietic cells is a prerequisite for its biological activity.

Materials and Methods

Transgene construction and microinjection

A 0.5-kb cDNA encoding p19 was cloned as an *Eco*RI fragment into an expression vector containing the human CMV enhancer/chicken β-actin promoter and the rabbit β-globin polyadenylation signal (7). For liver specific expression, the cDNA for p19 was cloned into an expression vector containing the promoter for human α_1-antitrypsin (HAT,[2] bp 1–1976, GenBank accession number K02212) and 145 bp of a transcriptional enhancer from the human α_1-microglobulin/bikunin gene (bp 2163–2308) (Fig. 5*A*). Transgenes were separated from vector sequences by zonal sucrose gradient centrifugation as described (8). Fractions containing the transgenes were pooled, microcentrifuged through Microcon-100 filters (Amicon, Bedford, MA), and washed five times with microinjection buffer (5 mM Tris-HCl (pH 7.4), 5 mM NaCl, 0.1 mM EDTA).

Generation of transgenic mice

DNA containing the transgene was resuspended in microinjection buffer to a final concentration of 1–5 ng/ml and microinjected into mouse eggs (C57BL/6J × DBA/2 (B6D2) F_2; The Jackson Laboratory, Bar Harbor, ME). The surviving eggs were transplanted into oviducts of ICR (Sprague Dawley) foster mothers, according to published procedures (9). By 10 days of life, a piece of tail from the resulting animals was clipped for DNA analysis. Founders carrying the p19 gene under control of the human CMV enhancer/chicken β-actin promoter (Cp19) were identified by PCR amplification of a segment of the transgene using primers 5′-GCCCTCCTC CAGCCAGAGGAT-3′ and 5′-CCAGCCACCACATTCTGATAGGCAGC CTGCAC-3′. As an internal control for the amplification reaction, primers for the endogenous low density lipoprotein gene were used: 5′-ACCCAA

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[2] Abbreviations used in this paper: HAT, human α_1-antitrypsin; SAA, serum amyloid A; IGF-1, insulin-like growth factor-1; H&E, hematoxylin and eosin; EMH, extramedullary hemopoiesis; AGP-1, α_1-acid glycoprotein.

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GACGTGCTCCCAGGATGA-3′ and 5′-CGCAGTGCTCCTCATCTGAC TTGT-3′. These primers amplify a 200-bp segment of the transgene and a 397-bp segment of the low density lipoprotein gene, respectively. To identify founders carrying the p19 gene under control of the HAT promoter (Hp19), we used the PCR primers 5′-CCGTTGCCCCTCTGGAT-3′ and 5′-GATTCATATGTCCCGCTGGTG-3′. As an internal control for this amplification, reaction primers for the endogenous *ZP3* gene were used: 5′-CAGCTCTACATCACCTGCCA-3′ and 5′-CACTGGGAAGAGACA CTCAG-3′. These primers amplify a 327-bp segment of the transgene and a 511-bp segment of the *ZP3* gene, respectively. PCR conditions were: 95°C, 30 s; 60°C, 30 s; 72°C, 60 s, for 30 cycles. The resulting transgenic mice were kept under specific pathogen-free conditions. Experiments were performed following the guidelines of the Schering-Plough Animal Care and Use Committee.

RNA analysis

RNA was extracted from tissues using RNA STAT-60, following specifications from the manufacturer (Tel-Test, Friendswood, TX). Total RNA (20 μg) was denatured and blotted onto Biotrans membrane (ICN Biomedicals, Costa Mesa, CA). Transgene expression was assessed by hybridization to randomly labeled (Stratagene, La Jolla, CA) p19 cDNA. Acute phase gene expression in liver was assessed by Northern blot hybridization of total RNA with ^{32}P-labeled PCR segments of the murine hemopexin gene (PCR primers: 5′-GGATGCCCGTGACTACCTTCGTAT-3′ and 5′-GGGCCAGGAAACCTCTGT-3′), of the murine α_1-acid glycoprotein (AGP-1; PCR primers: 5′-CCAGTGTGTCTATAACTCCACCC-3′ and 5′-GACTGCACCTATCCTTTTTCCA-3′) or of the murine haptoglobin gene (PCR primers: 5′-AGAGTATAGCCCAACCCTTCC-3′ and 5′-GAGAATAGTACAGTGCCCGAGAA-3′). A PCR segment of the murine β-actin gene was amplified using specific primers purchased from Stratagene, labeled as described above, and used for Northern blot hybridization.

ELISA

ELISA kits for murine IL-2 (sensitivity, <3 pg/ml), murine IL-1β (sensitivity, <3 pg/ml), murine IFN-γ (sensitivity, <2 pg/ml), murine TNF-α (sensitivity, <5.1 pg/ml), and murine IL-12 p40 (sensitivity, <4 pg/ml) were purchased from R&D Systems (Minneapolis, MN). ELISA kits for murine IL-6 (sensitivity, <8 pg/ml) and murine serum amyloid A (SAA; sensitivity, <0.23 μg/ml) were purchased from Biosource International (Camarillo, CA). ELISA kits for murine IL-1α (sensitivity, <6 pg/ml) were purchased from Endogen (Cambridge, MA). Assays were performed according to the manufacturer's instructions.

Levels of insulin-like growth factor-1 (IGF-1) in mouse serum were determined using a RIA for human IGF-1 that also recognizes murine IGF-1. Serum samples were acid-ethanol extracted according to instructions provided by the manufacturer (Nichols Institute, San Juan Capistrano, CA).

Histology

Mouse tissues were fixed by immersion in 10% phosphate-buffered formalin. Formalin-fixed tissues were routinely processed at 5 μm and were stained with hematoxylin and eosin (H&E). The number of megakaryocytes in transgenic and nontransgenic spleens was determined by counting the number of megakaryocytes in at least five different optical fields for each spleen.

Hematology

Blood samples were collected from the infraorbital sinus into sterile, evacuated tubes with added EDTA (Vacutainer Systems, Becton Dickinson, Rutherford, NJ). Hematological values were determined with an automated system (Cell-Dyn 3500; Abbott Laboratories, Abbott Park, IL). Platelet counts were performed manually when the instrument was unable to provide accurate platelet counts due to excessive clumping or excessively large platelets. Blood smears were stained with modified Wright-Giemsa stain (Hema-Tek Stain Pack; Bayer, Elkhart, IN) using an automated stainer (Hema-Tek 2000; Bayer). Bone marrow smear were obtained from the sternum, fixed in methanol, and stained with Wright-Giemsa stains using a Midas II stainer (EM Diagnostics Systems, Gibbstown, NJ).

Bone marrow transfers

Bone marrow cells were flushed from femurs of transgenic and control mice under sterile conditions. Single-cell suspensions (10 million cells) were injected into the tail veins of lethally irradiated (1100 rad for 5 min) B6D2F$_1$ mice (6–8 wk old).

Results

Animals expressing p19 in multiple tissues are runted, are infertile, and die prematurely

To express p19 in transgenic animals, we initially used the Cp19 transgene (Fig. 1*A*). The enhancer/promoter cassette used directs expression of the transgene to virtually all organs (10). Twenty-four founder mice (referred to as Cp19) were generated; of these, 10 died during the first 3 wk of life before data could be collected. Of the remaining 14 founders, 3 grew normally but 11 were small and failed to thrive (Fig. 1*B*). Reduced body weight was evident in the first 10 days of life (average weight for Cp19, 5.1 ± 1.9 g, n = 8; controls, 8.6 ± 1.4g, n = 23; p = 0.0007). Four of the 11 small founders (founders 25, 36, 51, 88) were severely runted, appeared moribund, and were sacrificed before weaning. To determine whether there was a correlation between the appearance of this phenotype and transgene expression, we performed Northern blot analysis of skeletal muscle RNA (Fig. 1*C*). Transgene expression was detected in all 11 Cp19 founders showing stunted growth, whereas no expression was detected in controls or in the 3 remaining, normally growing founders. All seven expressing Cp19 founders that survived beyond weaning age were clearly affected by the expression of the transgene; all had impaired growth, a swollen abdomen, and ruffled fur. These animals were infertile, and none survived beyond 90 days of age. Thus, ubiquitous expression of p19 resulted in stunted growth, infertility, and death.

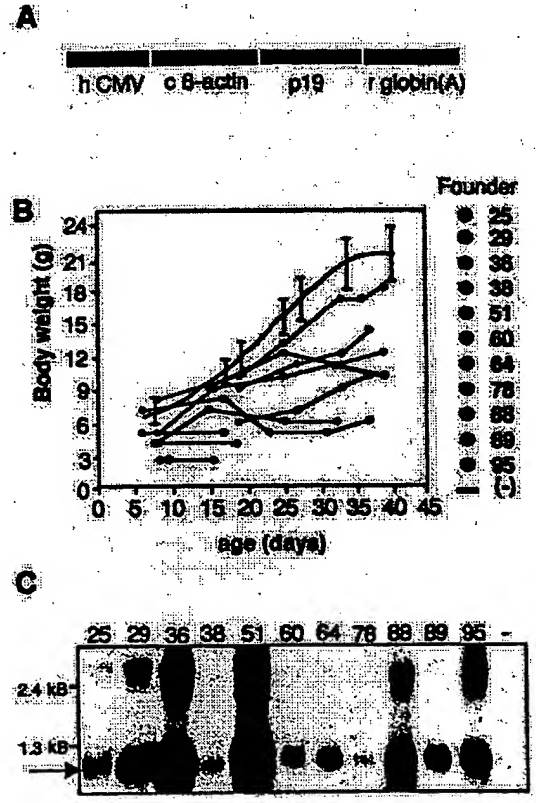

FIGURE 1. Impaired growth of Cp19-transgenic animals. *A*, Schematic representation of the Cp19 transgene with the human CMV enhancer (hCMV), chicken β-actin promoter (c β-actin), the murine p19 cDNA, and the rabbit β-globin polyadenylation signal (r globin(A)). *B*, Small body weight and stunted growth of Cp19-transgenic mice. Data were collected from 11 expressing Cp19 founders and from 69 nontransgenic littermates (−). Error bars, SD. *C*, Northern blot analysis of skeletal muscle RNA from transgenic founders and nontransgenic littermates (−) hybridized with p19 cDNA. The arrow indicates the major transcription product of the transgene.

Cp19-transgenic mice develop inflammation in multiple tissues and increased extramedullary hemopoiesis (EMH)

Tissues were collected from 10 of the 11 Cp19 founders described in Fig. 1, routinely fixed, processed to slides, and examined microscopically. Tissues collected from age- and gender-matched nontransgenic littermates were used as reference. Minimal to moderate inflammation (Fig. 2), sometimes associated with epithelial hyperplasia, was detected in all 10 Cp19 animals (age 15–85 days) in one or multiple sites, including the esophagus, stomach, small intestine, large intestine, skin, lungs, liver, and pancreas. In general, the inflammatory infiltrates consisted of lymphocytes and macrophages, sometimes accompanied by varying numbers of neutrophils. In the esophagus (Fig. 2, *A* and *B*), stomach, and intestines (Fig. 2*C*), the infiltrates were minimal to moderate, multifocal, and primarily localized in the epithelium, lamina propria, and submucosa and were often associated with epithelial hyperplasia. The hyperplasia resulted in lengthening of intestinal glands and shortening or loss of villi in the small intestine (Fig. 2*C*). Inflammation in the skin was multifocal, involved the epidermis and dermis, and was sometimes associated with acanthosis and/or ulceration (Fig. 2*D*). In the lungs (Fig. 2, *E* and *F*), findings consisted f peribronchial/perivascular mononuclear cell infiltrates; neutrophils were n t a prominent component of the pulmonary inflammation. In addition to the inflammation, the epithelium lining bronchi and bronchioles often had minimal to mild hyperplasia, sometimes with eosinophilic intracytoplasmic material. Inflammation in the liver consisted of minimal to mild periportal inflammatory infiltrates (data not shown). Pancreatic inflammatory infiltrates were minimal and consisted primarily of lymphocytes (data not shown).

In addition to inflammation, a few additional changes were noted. Minimal to mild EMH was observed in the liver and in medullary cords of lymph nodes, and mild to marked EMH was observed in the spleen of all 10 Cp19 founders examined (Fig. 2*H*). The splenic capsule was sometimes thickened (Fig. 2*H*). The cortex of lymph nodes was sometimes sparsely cellular and lacked secondary follicles. Microscopic changes were not observed in the skeletal muscle, heart, kidney, and brain, despite high levels of transgene expression in these organs (Fig. 1 and data not shown).

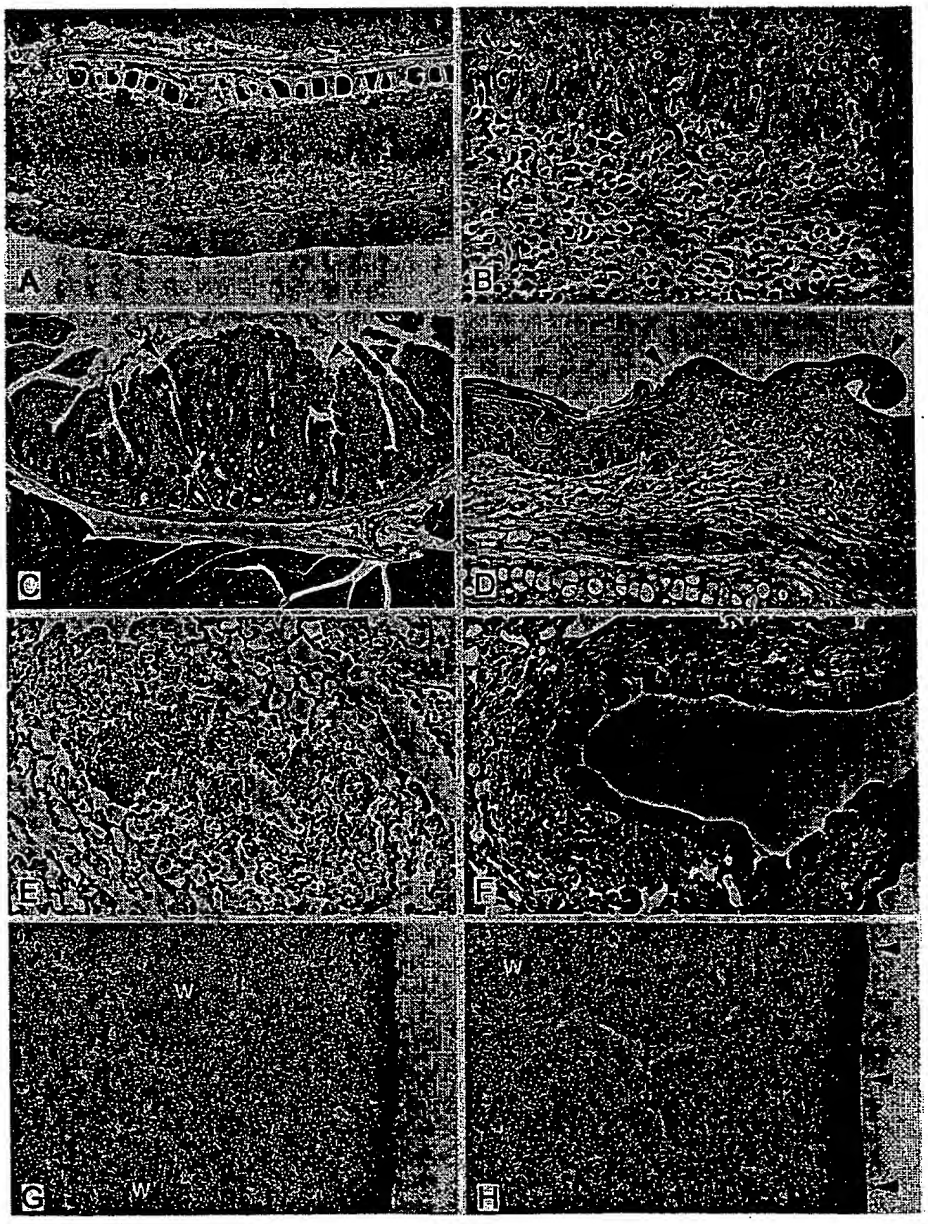

FIGURE 2. Multiorgan inflammation in Cp19 transgenic mice. *A*, Transgenic esophagus (magnification, ×80). Inflammation in the epithelium, lamina propria, and submucosa. *B*, Higher magnification (×320) of *A*, showing a mixture of lymphocytes, macrophages, and neutrophils in the inflammatory infiltrate. *C*, Small intestine from p19-transgenic mouse (magnification, ×40). Arrowheads, Focal inflammation with epithelial hyperplasia. *D*, Transgenic skin from ear (magnification, ×160). Lymphocytes, macrophages, and neutrophils in the dermis and epidermis, with focal epithelial necrosis and ulceration (arrowheads). *E*, Transgenic lung (magnification, ×160). Perivascular lymphocytes and macrophages. *F*, Transgenic lung (magnification, ×180). Peribronchial infiltrate of lymphocytes. There is also slight epithelial hyperplasia. *G*, Wild-type spleen (magnification, ×80) showing normal red and white (W) pulp. *H*, Transgenic spleen (magnification, ×80). Marked extramedullary hemopoiesis throughout the expanded red pulp. Only a small amount of white pulp is visible in this field because of the expanded red pulp. The capsule is also thickened (arrowheads).

Cp19 mice are anemic and have increased numbers of neutrophils in the peripheral blood

The effect of p19 on peripheral blood was analyzed in three independent founders and compared with data from three nontransgenic littermates. All Cp19 mice examined had mild to moderate microcytic hypochromic anemia. The mean hematocrit values in the Cp19 animals were 37–70% lower than that of controls (Cp19, 23.9 ± 8.3%; controls, 47 ± 2.4%, $p = 0.029$). Microscopic analysis f blood smears revealed erythrocytes of abnormal shape (poikilocytosis) and/or fragments of erythrocytes (schistocytosis). Slight to moderate polychromasia and variation in the size of erythrocytes (anisocytosis) suggested the presence of regeneration. The presence of microcytosis (small average RBC size) and hypochromia (diminished erythrocyte hemoglobin concentration) could be a direct effect of p19 activity or a secondary effect of the multiorgan inflammation in these animals.

The number of neutrophils in the blood was increased 3- to 11-fold over the highest neutrophil count in control animals (Table I). This increase in peripheral blood neutrophils, which is typical of inflammation, was associated with infiltration of neutrophils into various tissues. Accordingly, the myeloid component (as assessed by the granulocytic/erythroid ratio) was increased in the bone marrow relative to the erythroid component. Interestingly, the number of circulating platelets in Cp19 animals was slightly increased over the number found in control littermates (Cp19, 1708 ± 599; control, 821 ± 32 × 10^3 cells/μl; $p = 0.12$). Although these differences were not statistically significant, the presence of excessive clumping and platelets of unusual morphology in Cp19 blood smears suggested increased platelet production. To determine whether the increased number of platelets in Cp19 founders was caused by accelerated platelet production by megakaryocytes or by an increase in the number of megakaryocytes, we examined the spleen and bone marrow of Cp19 mice microscopically. In these tissues, megakaryocytes were enlarged, but their numbers were not increased (Cp19 ($n = 11$), 7.5 ± 5.8; control ($n = 5$), 8.1 ± 3.2 per optical field; $p = 0.81$), suggesting that the mild thrombocytosis observed in Cp19 blood resulted from accelerated production of platelets by megakaryocytes.

Cp19-transgenic mice express high levels of proinflammatory cytokines, but not IL-6

T test whether the systemic inflammation seen in Cp19 animals was associated with altered expression of proinflammatory cytokines, we determined the concentrations of IL-1, TNF-α, IL-6, IFN-γ, and IL-12p40 in the peripheral blood. Concentrations of TNF-α were significantly increased in 7 of 10 founders tested (Fig. 3), and concentrations of IL-1 were increased in 2 of 3 founders tested (292 and 518 pg/ml). IL-1 concentrations in serum of unchallenged control littermates ranged from 0 to 70 pg/ml. The levels of TNF-α and IL-1 observed in Cp19 mice were within the range of those observed in control mice after induction of acute inflammation by LPS (M. T. Wiekowski, unpublished observation).

Table I. *Analysis of leukocyte populations in blood of Cp19 and control animals*[a]

Cell Type	Controls	Cp19 Founders
Neutrophils	0.60 ± 0.14	4.71 ± 3.24*
Lymphocytes	4.91 ± 1.40	2.79 ± 1.47*
Monocytes	0.29 ± 0.24	0.69 ± 0.6
Eosinophils	0.11 ± 0.02	0.09 ± 0.04
Basophils	0.002 ± 0.005	0.09 ± 0.11

[a] Values are expressed as 10^3 cells/μl. Results for Cp19 founders ($n = 3$) and nontransgenic controls ($n = 3$) are expressed as mean ± SD. *, $p < 0.05$.

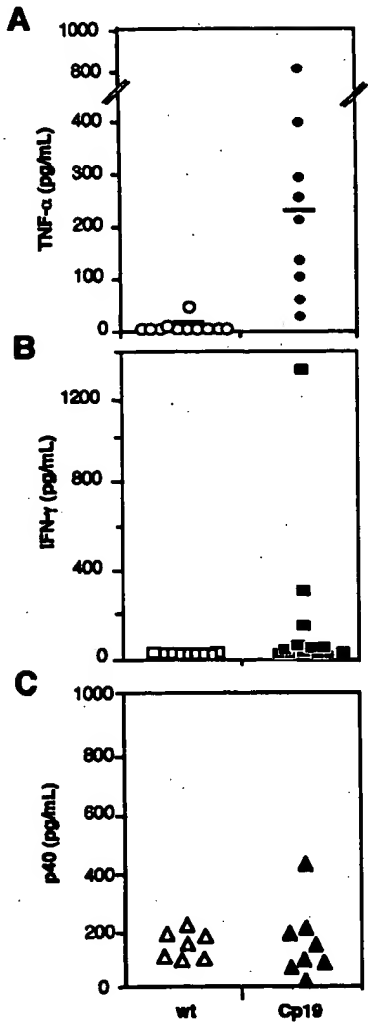

FIGURE 3. Cytokine expression in Cp19 founders. Concentrations of TNF-α (*A*), IFN-γ (*B*) and p40 (*C*) were determined in serum of Cp19 founders and wild-type (wt) animals by ELISAs specific for the selected cytokine. Bars in *A* represent the average TNF-α concentrations found in wild-type and Cp19 animals ($p = 0.008$).

In addition, concentrations of IFN-γ were increased in 3 of 13 founders tested (Fig. 3). Another cytokine involved in an inflammatory response is IL-12, a heterodimeric cytokine formed by association of the subunits p40 and p35 (6, 11, 12). Therefore, we determined the concentration of p40 in serum of eight Cp19 founders. With the exception of a single Cp19 founder, the levels of p40 did not differ from controls (Fig. 3*C*). This indicated that overexpression of p19 in Cp19 animals did not elevate the concentrations of circulating p40.

Surprisingly, IL-6 protein could not be detected in the peripheral blood of Cp19 animals, despite the high circulating concentrations of TNF-α and IL-1, cytokines that directly induce IL-6 production (13). This was especially surprising considering the fact that IL-6 is expressed during systemic inflammation (14, 15).

Acute phase protein genes are chronically expressed in the livers of Cp19 animals

During inflammation, genes encoding acute phase proteins are upregulated in the liver. Because Cp19 mice exhibit a phenotype characterized by systemic inflammation, we investigated whether

the expression of acute phase genes was altered in their livers. As shown in Fig. 4*A*, the acute phase genes AGP-1, haptoglobin, and hemopexin were highly expressed in the liver of all four transgenic f unders tested, whereas no expression of these genes was detected in nontransgenic livers. T test whether the concentration of acute phase proteins was also increased in the circulation, blood from Cp19 founders was tested for the presence of SAA. The average level of circulating SAA (248 ± 159 μg/ml, $n = 10$) was significantly increased over the levels found in controls (8 ± 5.1 μg/ml, $n = 8, p = 0.05$) (Fig. 4*B*). These results indicate that acute phase liver genes are chronically expressed in Cp19 animals.

Impaired growth and infertility of Cp19 animals are associated with decreased circulating concentrations of IGF-1

Growth impairment caused by chronic inflammatory conditions (16, 17) or by overexpression of cytokines in transgenic animals (18) is associated with a decrease in the circulating levels of IGF-1, a hormone that regulates postnatal growth (19) and influences fertility (20). To test whether the impaired growth of Cp19 animals was associated with reduced levels of IGF-1, serum samples of transgenic animals were assayed for IGF-1. In all founders tested, the amount of IGF-1 in the serum was reduced to 12–14% of the concentrations found in nontransgenic, age-matched littermates (Table II). This suggests that overexpression of p19 may directly or indirectly reduce IGF-1 concentrations, resulting in impaired growth and infertility of transgenic animals (20).

Liver-specific expression of p19 in transgenic mice does not result in a detectable phenotype

The infertility and premature death seen in Cp19 transgenic animals precluded further analysis of the biological function of p19. Therefore, we attempted to generate another transgenic model expressing p19 using a tissue-specific promoter. To this end, we made transgenic animals carrying the p19 gene under the control of the liver-specific human α_1-antitrypsin promoter (Hp19 animals). We generated eight founders, from which seven transgenic lines were derived. Transgene expression was detected by Northern blot analysis in all mice analyzed, but not in controls (Fig. 5*B*).

Table II. *Serum IGF-1 levels are reduced in p19-transgenic mice*[a]

Founder	Age (days)	IGF-1 (ng/ml)	% of Control
36	15	25	12.1
89	31	28	12.2
60	54	59	14.5

[a] Absolute levels of IGF-1 in serum of transgenic mice were determined and compared with those of age-matched control littermates ($n = 6$).

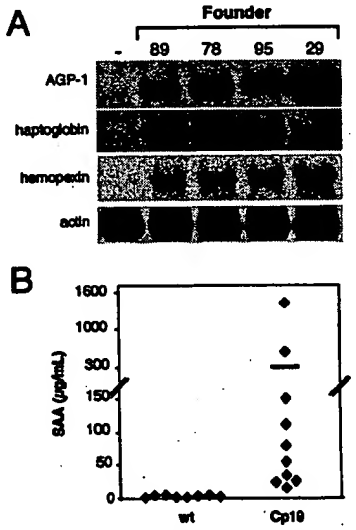

FIGURE 4. Expression of acute phase liver genes in Cp19 animals. *A*, Total RNA (20 μg) extracted from the liver of Cp19-transgenic animals (founders 89, 78, and 95; 35–85 days old) and nontransgenic littermates (−) was probed with radiolabeled PCR fragments for the murine genes AGP-1, hemopexin, and haptoglobin. Equal loading of RNA for each sample was verified by reprobing with a radiolabeled PCR fragment for the murine β-actin gene after the blot had been stripped. *B*, Levels of the acute phase protein SAA in serum of 10 Cp19 founders (age 16 days–3 mo) and nontransgenic animals (wild-type (wt), $n = 8$; $p = 0.05$). Bar represents the average SAA level in Cp19 animals.

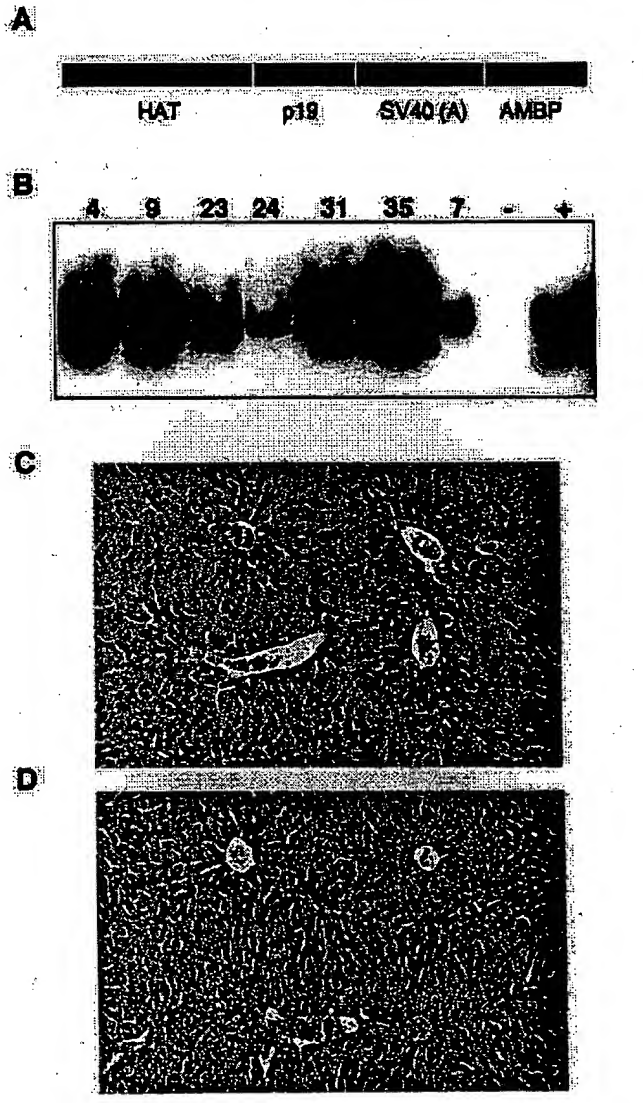

FIGURE 5. Transgenic expression of p19 in liver does not lead to inflammation. *A*, Schematic representation of the Hp19 transgene with HAT, the SV40 polyadenylation signal (SV40 (A)) and a transcriptional enhancer from the human α_1-microglobulin/bikunin gene (AMBP). *B*, Northern blot analysis of liver RNA from Hp19-transgenic lines and nontransgenic littermates (−) hybridized with cDNA for p19. For comparison, skeletal muscle RNA from a Cp19 founder (+) was included. *C* and *D*, H&E-stained liver from control (*C*) and transgenic (*D*) Hp19 animal (magnification, ×75). Normal appearance of liver with central vein (arrows) and several portal areas.

The two highest expressing lines were expanded, and a detailed analysis of growth patterns, blood parameters, expressi n of acute phase genes, and histology of several organs was performed. Surprisingly, these transgenic animals grew normally and had n signs f hepatic (Fig. 5, *C* and *D*) or systemic inflammation (data not shown). In addition, we were unable to detect any changes in the peripheral blood cell composition or in the expression of acute phase genes (data not shown). These results suggest that there are requirements for the biological activity of p19 that are not satisfied by its production in liver cells.

Transplantation of transgenic hemopoietic cells into wild-type mice results in the development of multiorgan inflammation

The absence of a phenotype in mice overexpressing p19 in liver and the observation by Oppmann et al. (1) that p19 is expressed by a subset of hemopoietic cells led us to examine whether overexpression of p19 by hemopoietic cells would be sufficient to induce a phenotype similar to the one described for Cp19 animals. We bserved previously that the CMV/β-actin promoter targets expression of transgenes to a variety of cells within the immune system, including T and B lymphocytes and dendritic cells (21). Thus, we generated a new set of Cp19 founders and transferred their bone marrow (Fig. 6*A*) into lethally irradiated wild-type recipient mice. The health of these Cp19 bone marrow recipients deteriorated within 35–66 days posttransfer, as judged by the appearance of ruffled fur and inflamed skin around the snout and ventral neck. In contrast, recipients of wild-type bone marrow did not develop an obvious phenotype. Analysis of the bone marrow recipients showed p19 expression in the bone marrow (data not shown) and spleens of Cp19, but not control, bone marrow recipients (Fig. 6*A*). In Cp19 bone marrow recipients, the acute phase liver genes hemopexin and AGP-1 were highly expressed (not shown), and the serum levels of SAA were elevated (Fig. 6*B*), but again no IL-6 could be detected in circulation (data not shown). As in Cp19 donor animals, skin, lung, liver, and the gastrointestinal tract were inflamed in recipients of Cp19 bone marrow, but not in wild-type bone marrow recipients. Perivascular/peribronchial infiltrates of lymphocytes and macrophages and slight epithelial hyperplasia were observed in the airways of Cp19 bone marrow recipients (Fig. 6, *C* and *D*). These results indicate that p19 produced by bone marrow cells is biologically active and can induce a phenotype of systemic inflammation similar to that obtained by ubiquitous expression of p19.

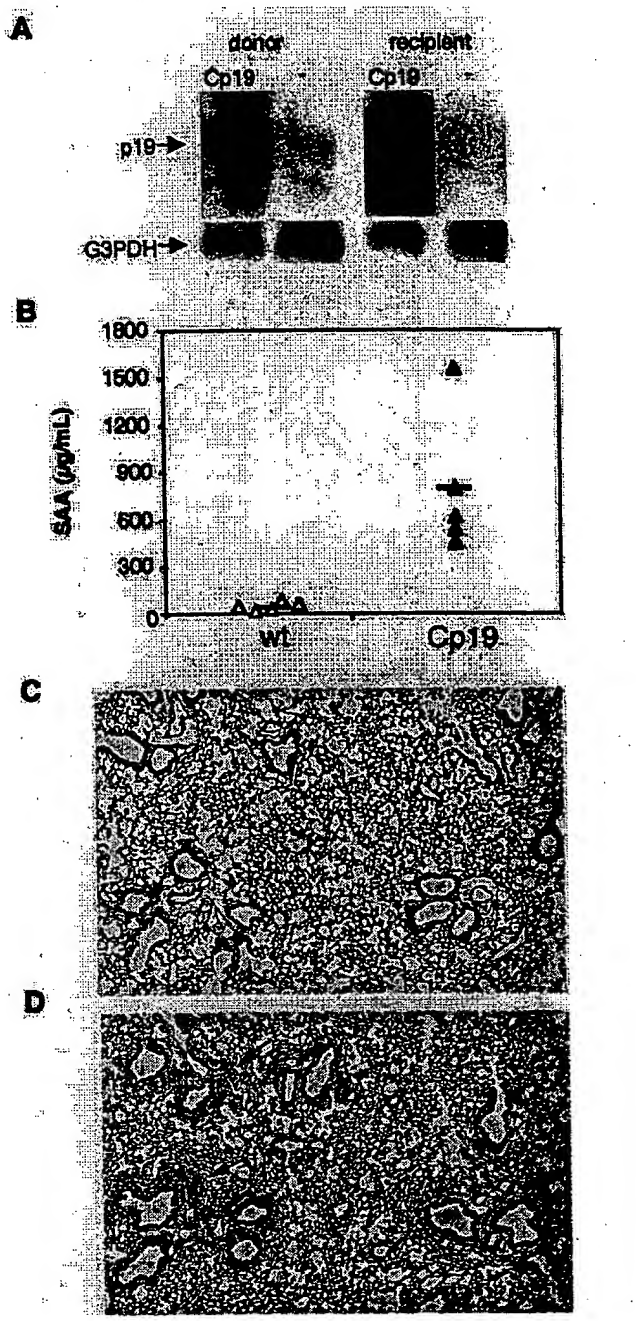

FIGURE 6. Inflammation and an acute phase response in wild-type recipients of Cp19-transgenic bone marrow. *A*, Northern blot analysis of p19 expression in spleens of donor animals for Cp19-transgenic and nontransgenic bone marrow (−) and recipients of bone marrow from Cp19 or nontransgenic (−) bone marrow. *B*, Levels of the acute phase protein SAA in serum of recipients of Cp19 bone marrow (Cp19; $n = 5$) or wild-type bone marrow (wt; $n = 5$). Bars represent the average level of SAA in each group. *C* and *D*, H&E-stained lung from nontransgenic (*C*) and Cp19 transgenic (*D*) bone marrow recipients (magnification, ×37.2). Arrows show perivascular/peribronchial infiltrates of lymphocytes and macrophages. There is also slight epithelial hyperplasia in the airways.

Discussion

T characterize the biological activity of p19, we expressed it in transgenic mice. Widespread expression of p19 led to a phenotype of systemic inflammation, impaired growth, and premature death. Tissue-specific expression of p19 yielded two outcomes. Animals expressing p19 in liver were fertile, had a normal life span, and did not present signs of systemic or localized inflammation. In contrast, animals expressing p19 in bone marrow-derived cells presented a phenotype of systemic inflammation that was similar to the ne observed when p19 was expressed ubiquitously. These results indicate that hemopoietic expression of p19 is necessary and sufficient to induce systemic inflammation, impaired growth, and premature death, phenotypes that are strikingly reminiscent of those observed in mice overexpressing cytokines structurally related to p19 (Table III). For example, runting or failure to thrive has been reported for transgenic animals expressing IL-6 in skin (22), astrocytes (23), neurons (18), and in the airway epithelium (24); and for transgenic animals expressing oncostatin M in the skin (25). Similar to observations in mice overexpressing IL-6, Cp19 mice had reduced levels f IGF-1, which may have contributed to their failure to thrive and their reduced fertility (18). It is unclear whether the reduced levels f IGF-1 were directly caused by p19 or caused by other factors secreted during systemic inflammation.

Other striking findings in Cp19 animals were the inflammation affecting multiple organs and the high levels of circulating TNF-α

Table III. *Comparison of phenotypes of p19-, IL-12-, IL-6-, and G-CSF-transgenic mice*

Phenotype	p19	IL-12	IL-6	G-CSF	Ref.
Inflammation	Yes	Yes	Yes[a]	NR[b]	12, 26, 37
Impaired growth	Yes	NR	Yes	NR	22–24
Anemia	Yes	NR	Yes	NR	32
Reduced IGF-1 levels	Yes	NR	Yes	NR	38
Acute phase response	Yes	NR	Yes	NR	23, 26, 39, 40
Thrombocytosis	Yes[c]	NR	Yes	NR	26
Neutrophilia	Yes	NR	NR	Yes	5, 26

[a] In IL-6-transgenic animals, inflammation is observed only upon transgene expression in liver or plasma cells.
[b] NR, Information was not reported.
[c] No significant increase in platelet numbers ($p > 0.05$).

and IL-1. Similar findings have been reported in transgenic mice expressing the p35/p40 IL-12 heterodimer in brain (12). Inflammati n, usually in the form of mesangioproliferative glomerulonephritis, has also been observed in mice overexpressing IL-6 in liver (26) and plasma cells (27). This condition, however, usually develops after several months of chronic exposure to IL-6, presumably as a function of the high circulating levels of Igs. This localized inflammation differs from the systemic inflammation observed in CP19 mice, suggesting that the mechanisms mediating inflammation in the Cp19 mice may be different from those induced by IL-6.

Neutrophilia has also been shown as a result of transgenic expression of IL-6 (26) and G-CSF (5, 28), another closely related cytokine. However, G-CSF-induced neutrophilia was not associated with other phenotypic changes as described here for Cp19 animals. Interestingly, the degree of EMH observed in the spleen f Cp19 animals was disproportionate to the systemic inflammation seen in these animals, raising the intriguing possibility that p19, like G-CSF, may have a direct effect on hemopoiesis (29). Likewise, a direct negative effect of p19 on erythropoiesis cannot be ruled out, because the Cp19 mice are anemic. Anemia has also been observed in animals overexpressing IL-6 (32) and in animals repeatedly injected with IL-12 (11). An indirect mechanism contributing to the anemia observed in the Cp19 mice could be the dysregulated cytokine production. For instance, TNF-α and IL-1, cytokines that are elevated in the Cp19 mice, have been shown to play a role in anemia of chronic disorders (35, 36), a microcytic hypochromic anemia often seen in humans with inflammatory disease.

In Cp19 animals, acute phase liver genes like AGP-1, haptoglobin, hemopexin, and SAA were chronically expressed, and platelet production was increased. Because IL-6 is the primary inducer of an acute phase response (reviewed in Ref. 30) and functions as a megakaryocyte differentiation factor (31, 32), the phenotype described here could be caused by p19-mediated up-regulation of IL-6 expression. However, neither IL-6 protein nor IL-6 mRNA (data not shown) could be detected in Cp19-transgenic mice. These results suggest that p19 may function directly in the induction of an acute phase response and megakaryocyte differentiation.

In vitro, p19 is secreted in complex with the p40 subunit of IL-12. This p19-p40 heterodimer has now been named IL-23 (1). Apparently, this association is not only necessary for secretion but also seems essential for its biological function, because purified p19 is biologically inert in vitro. These findings have important implications for the interpretation of the phen types described here. Expression f p40 is normally restricted to monocytes, macrophages, and dendritic cells in the mouse (33). We hypothesize that the phenotype observed in mice expressing p19 in hemopoietic cells is the function of the simultaneous production of p40, and we suggest that the absence of a phenotype in the animals expressing p19 in liver is due to lack of coexpression of p40 (34). Our inability to detect p40 in the serum of Cp19 animals may indicate either that p19/p40 complexes are found at very low levels in circulation or that they act locally, in an autocrine or paracrine manner. It is also possible that p19 produced by hemopoietic cells has biological activities that are independent of its dimerization with p40.

TNF-α and IL-1 are also known inducers of IL-6 expression (13). Thus, it was surprising that no IL-6 could be detected in Cp19 animals despite the high concentrations of circulating TNF-α and IL-1. This intriguing and unexpected result suggests that p19 may have a negative effect on IL-6 expression by a yet unidentified mechanism.

Overexpression of p19 in Cp19 animals did not always result in an increase of IFN-γ expression. This in vivo result differs from the in vitro results obtained by Oppmann et al. (1), who reported induction of IFN-γ by T-cells after IL-23 (p19/p40) treatment. Unfortunately, this discrepancy could not be satisfactorily resolved because of the short life span of the transgenic mice, which precluded analysis of specific immune responses. Thus, the function of p19 on expression of IFN-γ in vivo and its functional relationship to IL-12 remains to be determined in mice deficient for p19 and in transgenic mice expressing p19 conditionally.

Our results show that overexpression of p19 in vivo induces a phenotype resembling that observed on overexpression of the structurally related cytokines IL-12, IL-6, and G-CSF. Further studies will be necessary to understand how expression of p19 leads to these phenotypes and the molecular nature of the receptor(s) mediating these responses.

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